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Effect of Feeding Pyridine Derivatives to Young Rats on a High Protein Diet.

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The inhibition of the growth of young rats on a low protein diet by nicotinamide but not by nicotinic acid at a dietary level of 1% led Handler and Dann[1] to postulate that the more rapid methylation of nicotinamide resulted in a greater decrease in the available "methyl donors" than that which occurred following ingestion of nicotinic acid. They were able to demonstrate that the administration of methionine to rats fed large amounts of nicotinamide resulted in an increase in the "trigonelline"* fraction of the urine and an increase in the growth rate. However, the need for methylation in the process of detoxication does not fully explain the reason for the high toxicity of coramine (nikethamide) which is much more slowly methylated than even the relatively nontoxic nicotinic acid.[2,3] It is possible that much

1 Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **146**, 337.

* This "trigonelline" fraction has since been shown to consist chiefly of nicotinamide methochloride (N1 methyl nicotinamide).

2 Ellinger, P., and Coulson, R. A., *Biochem. J.*, 1944, **38**, 265.

of the toxicity of pyridine, nicotinamide and coramine is inherent in the molecular structure and is not due solely to any secondary effect such as the depletion of the store of methionine or choline since the subcutaneous administration of these compounds in relatively small doses leads to immediate manifestations of toxicity.[4]

This communication reports the influence of the oral administration of 17 pyridine derivatives on rat growth and on the weight and on some aspects of the composition of the rat liver.

Experimental. Preparation of Basal Rat Diets. For the entire series of experiments the rats were maintained on a comparatively high protein diet which consisted of cottonseed oil, 15, casein, 25, salts,[5] 5 and starch, 55 parts. The high protein diet was adopted as a protective measure inasmuch as many of the compounds are quite toxic. Experimental diets were prepared by inclusion of the appropriate supplement in the basal diet. Each rat also received 1.0 ml of Brewers' yeast extract a day (equivalent to 1 g of dry yeast) and 1 drop of Percomorph oil a week. The experimental animals were an inbred mixture of the Sprague-Dawley and Illinois strains. To avoid any difficulties due to sexual differences equal numbers of males and females initially weighing from 45-50 g were utilized for each group. In no instance were 2 rats from the same litter used in the same experimental group.

Effect of the Methylated Pyridine Nucleus on the Growth Rate. His[6] reported the isolation of methyl pyridinium hydroxide from the urine of a dog which was fed repeated doses of pyridine. Since this publication in 1887 it has been generally supposed that pyridine is methylated in the process of "detoxication" and eliminated in the urine as the methyl derivative.

Experiments were devised to test the effect of feeding several methylated and non-methylated pyridine derivatives to young rats. Groups of rats were fed nicotinic acid, nicotinamide, coramine, alpha, beta and gamma picoline, isonicotinic acid and alpha picolinic acid at levels of 1% of the diet for 28 days. The respective N-methyl derivatives of these compounds were synthesized by methylating the parent substance with methyl iodide or dimethyl sulphate followed by the subsequent conversion to the chloride salt through treatment with silver chloride. These methylated compounds were recrystallized several times and added to the basal diet at concentrations which were equivalent to 1% of their immediate precursors on the basis of their respective molecular weights.

Whereas methylation of some of the pyridine derivatives resulted in a decrease in the degree of growth inhibition (Table I) other compounds were unaffected or actually rendered more toxic by this procedure. In general, the rats grew at a rate which was roughly proportional to their food intake regardless of the compound administered. This would indicate that although the appetite decreased these substances had no effect on the intestinal absorption or the assimilation of the diet. There was little correlation between the acute toxicity as measured by subcutaneous injection[4] and the toxicity as measured by the degree of growth inhibition following prolonged administration by dietary means. The addition of choline or methionine to diets containing beta picoline or coramine failed to increase the growth rate significantly over that of rats which did not receive any lipotropic compound. It would appear that any growth inhibition caused by beta picoline or coramine is not reduced by the addition of substances that are necessary for transmethylation, or, that the process of methylation has little connection with the decreased appetite and the resultant growth decrease if an otherwise adequate high protein diet is fed.

Detoxication Products of Pyridine and Coramine. In an effort to determine whether the rat actually does methylate pyridine a group of rats were fed for 10 days a diet

[3] Coulson, R. A., and Stewart, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 364.

[4] Brazda, F. G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 19.

[5] Hawk, P. B., and Oser, B. L., *Science*, 1931, **74**, 369.

[6] His, W., *Arch. expt. Path. u. Pharm.*, 1887, **22**, 253.

TABLE I.
Effect of Oral Administration of Pyridine Derivatives to Young Rats on a High Protein Diet for 28 Days.

Compound %	No. rats	Avg final wt g	Avg liver wt wet g	Avg % solid	Avg liver wt wet as % body wt	Avg % fat wet wt	Avg % fat dry wt	Avg food intake g/day	Avg wt gains g/day
Pyridine methochloride 1.64%	6	113.5	5.05	35.05	4.45	4.30	12.39	5.55	2.26
Alpha picoline 1%	5*	110.8	4.96	38.03	4.29	3.77	10.91	4.84	2.34
Alpha picoline methochloride 1.54%	5†	50.6	2.09	30.62	4.17	1.76	5.78	1.91	0.50
Beta picoline methochloride 1.54%	6	97.3	3.66	31.53	3.70	3.42	11.38	4.00	1.91
Gamma picoline 1%	6	100.9	4.47	35.30	4.56	6.23	18.54	4.08	1.96
Gamma picoline methochloride 1.54%	6	92.0	3.42	28.32	3.77	2.25	7.62	3.69	1.69
Alpha picolinic acid 0.70%	5*	141.5	6.06	32.15	4.28	2.78	8.65	7.76	3.40
Alpha picolinic acid methyl betaine 1.12%	6	77.4	3.59	31.75	4.64	2.42	7.19	3.76	0.73
Nicotinic acid 1%	6	147.8	7.50	36.00	5.08	6.68	18.56	8.30	3.54
Trigonelline 1.12%	6	169.2	7.08	33.47	4.18	4.14	12.45	9.95	4.33
Isonicotinic acid 1%	8	131.8	5.20	34.04	3.92	4.19	12.43	6.79	3.07
Isonicotinic acid methyl betaine 1.12%	6	135.1	5.21	33.78	3.86	3.38	9.89	6.20	3.15
Nicotinamide 1%	8	105.6	5.12	34.57	4.85	6.89	19.96	5.98	2.18
Nicotinamide methochloride 1.41%	6	159.1	7.60	33.95	4.78	4.62	13.59	9.11	3.95
Coramine methochloride 1.28%	6	167.5	7.05	32.62	4.21	3.97	11.75	10.06	4.16
Control	12	155.5	6.59	33.82	4.25	4.16	12.36	8.18	3.91

The content of methylated pyridine compounds in the diet is equivalent, in each case, to a 1% content of the immediate unmethylated precursor.

* The 5 rats in these groups represent the survivors of the starting groups of 6.

† Alpha picoline methochloride proved to be too toxic to permit the experiment to continue for 28 days. This group represents the survivors of a 14-day experiment.

which contained 1% pyridine. The urine was collected and attempts were made to isolate pyridine methochloride by essentially following the procedure described by His.[6] No free pyridine was detected in the urine nor was it possible to isolate any of the methyl derivative. On the other hand, it was possible to isolate pyridine methochloride if the rats were fed this compound. Nicotinamide methochloride was isolated from the urine of rats which received nicotinic acid, nicotinamide, or coramine. We could find no evidence for the direct methylation of coramine to coramine methochloride. Similar studies involving the metabolism of the other compounds in this series are in progress and the results will appear in a subsequent publication.

It would be premature to conclude that coramine and pyridine are not directly methylated since the "detoxified" methyl derivative might be converted to some other substance before being eliminated by the kidney. However, it does seem unlikely that any considerable fraction of these compounds is methylated by the rat and it is probable that the rat uses some means other than methylation to reduce their concentration in the body. No information is available on the degree of absorption of these compounds from the intestine of the rat. However, coramine, nicotinic acid and nicotinamide are known to be quickly absorbed from the human intestine.[2]

Effect of Pyridine Derivatives on Liver Fat. The rats were killed at the end of a 28-day feeding period, the livers were removed and the water and total lipids were determined. A sample of each liver was saved and sectioned for histological examination. It is evident that the use of the high protein diet prevented the development of livers which were as fatty as those usually developed on a low protein diet. (Tables I and

TABLE II.

Effect of Addition of Choline or Methionine to Diet of Young Rats Receiving β-picoline or Coramine for 28 Days.

Compound %	No. rats	Avg final wt g	Avg liver wt wet g	Avg % solid	Liver wt wet as % of body wt		Liver fat as % wet liver wt		Avg % fat dry wt	Avg food intake g/day	Avg wt gains g/day
					Range of values	Mean	Range of values	Mean			
Beta picoline 1%	6	97.5	4.61	39.02	4.05 5.29	4.51±0.13*	5.33 21.10	11.33±1.44	29.95	4.00	1.87
Beta picoline 1%+ 0.5% choline	6	114.3	4.99	33.87	4.00 5.05	4.36±0.10	2.98 5.70	3.91±0.24	11.24	4.72	2.37
Beta picoline 1%+ 1.2% methionine	6	102.0	4.69	33.26	4.07 4.91	4.61±0.08	2.58 4.49	3.40±0.20	10.32	3.96	1.94
Coramine 1%	12	121.5	10.73	36.96	6.82 9.71	8.19±0.27	3.62 7.34	5.78±0.33	17.47	6.71	2.68
Coramine 1%+ choline 0.50%	6	108.8	7.88	32.23	6.01 7.80	7.06±0.16	1.87 3.47	2.38±0.14	7.40	5.69	2.32
Coramine 1%+ methionine 1.2%	6	117.1	9.17	34.13	6.68 9.56	7.83±0.27	2.89 5.35	3.62±0.22	11.76	5.69	2.58
Control	12	155.5	6.59	33.82	3.82 4.83	4.39±0.09	2.76 5.01	3.57±0.18	12.36	8.18	3.91

* Probable error of the mean result.

II). None of the methylated compounds produced any significant increase in the fat content of the liver as determined by gravimetric analysis or microscopic examination. In spite of the small number of rats in each group it is statistically improbable that any of these methylated compounds are capable of increasing liver lipids since none of the 53 rats that received a methylated compound showed any increase in liver fat. Gamma picoline, nicotinic acid, and nicotinamide produced some increase in the fat content (Table I) in spite of the high protein diet. The significance of this change is not clearly established. However, it has been demonstrated that nicotinic acid and nicotinamide definitely produce fatty livers in rats maintained on low protein diets.[1] Beta picoline and coramine produced a definitely significant increase in the liver fat content (Table II). There was good correlation between the histological appearance and the chemical analysis in all groups. Alpha picoline, alpha picolinic acid and isonicotinic acid did not produce any significant change in the fat content (Table I). The effect of pyridine, under these experimental conditions, will be published later.

Effect of Coramine on Liver Size. In addition to the increase in liver fat caused by coramine this compound invariably caused a great increase in the absolute nonfat liver weight. The average liver weight of the 24 rats that received coramine, either alone or supplemented with choline or methionine, was very nearly twice that of the controls. This increase is not due to hydration since the per cent solid content remains normal. None of the other substances reported in this study had any effect in this regard. The effects produced by beta picoline and coramine are compared in Table II. Although the addition of 0.5% choline or 1.2% methionine prevented the increase in liver fat† caused by either beta picoline or coramine the great increase in the nonfat weight

† Preliminary experiments with coramine indicated that neither 0.15% choline nor 0.6% methionine was able to prevent the increase in liver lipids.

due to coramine was not prevented by the addition of choline or methionine. The fact that a combination of lipotropic factors and high protein diet was not capable of preventing the abnormal liver growth would suggest that this action of coramine is not due to any decrease in factors necessary for transmethylation but to a specific action of the coramine molecule.

Summary. 1. The effect of the ingestion of a series of pyridine derivatives by immature rats on a high protein diet is described. 2. None of the N-methyl derivatives of this series had any appreciable influence on either the fat content or the absolute liver weight. 3. Gamma picoline, nicotinic acid, and nicotinamide produced slight increase in liver fat; beta picoline and coramine produced significant increases in liver fat. In the case of beta picoline and coramine this increase in fat could be prevented by the inclusion of 1.2% methionine or 0.5% choline. The effect of methionine or choline on the metabolism of the other compounds reported was not determined. 4. Coramine produced a great increase in the fat-free liver weight which could not be prevented by the addition of choline or methionine to the diet. 5. The growth inhibition following the administration of beta picoline or coramine was not appreciably affected by the addition of choline or methionine to the high protein diet.

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Growth and Reproduction in Rats on Synthetic Rations.*

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Introduction. It is now generally recognized that rats grow at a rapid rate when given synthetic diets containing only 6 B vitamins. When sulfonamides are added, the growth is greatly retarded but the effect is counteracted by the addition of folic acid and biotin. Ransone and Elvehjem[1] reported that certain folic acid concentrates from liver produced a growth response beyond that which could be attributed to the known vitamins present. Recently evidence has been presented for the presence in fresh liver and other materials, of a factor necessary for continued growth and a normal blood picture in the monkey.[2] Further work has shown the existence of an unknown factor(s) in liver which stimulates the growth of *S. faecalis* when this organism is cultured on a "complete" synthetic medium.[3,4] The present work was undertaken to determine the effect upon growth and lactation of rats receiving a purified ration containing 10 crystalline B vitamins.

Experimental. Male weanling rats (Sprague-Dawley) weighing between 38 and 45 g were used in all growth experiments. They were housed in individual cages and were fed the

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1 Ransone, B., and Elvehjem, C. A., *J. Biol. Chem.*, 1943, **151**, 109.

2 Cooperman, J. M., Ruegamer, W. R., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 101.

3 Cooperman, J. M., Ruegamer, W. R., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **163**, 769.

4 Ruegamer, W. R., Cooperman, J. M., Sporn, E. M., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **167**, 861.

TABLE I.
Effect of Liver Supplements on the Rate of Growth in Rats.

Group	Period wk	Supplement	Avg gain per wk g
		Basal ration	
1	4	None	29
2	4	5 cc raw milk per day	33
3	4	0.5 g " liver " "	36
4	4	3% lyophilized liver (Squibb)	38
5	4	1% 1:20 liver powder (Wilson)	35
		Basal ration + double level of B vitamins + cystine	
6	2	None	31
7	2	0.5 g raw liver per day	34
8	2	3% lyophilized liver (Squibb)	37
9	2	1% 1:20 liver powder (Wilson)	35
10	2	1% Lederle liver prep. No. 1432	36
		Corn-soybean meal ration + double level of B vitamins	
11	3	None	36
12	3	0.5 g raw liver per day	43
13	3	3% lyophilized liver (Squibb)	43
14	3	1% 1:20 liver powder (Wilson)	45
15	3	1% Lederle liver prep. No. 1432	44

basal ration and fresh water *ad libitum*. Six rats were used in each group.

The synthetic basal ration was composed of sucrose 73%, casein (Smaco) 18%, corn oil (Mazola) 5%, salts IV 4%, and thiamine 0.3 mg, riboflavin 0.3 mg, niacin 2 mg, pyridoxine 0.2 mg, pantothenic acid 2 mg, folic acid 0.025 mg, biotin 0.01 mg, inositol 10 mg, choline 100 mg, and *p*-aminobenzoic acid 25 mg per 100 g of ration. Vitamins A and D were administered as oleum percomorphum diluted 1:4 with corn oil and fed at the rate of 2 drops per week.

Since milk and liver had been shown to be good sources of the monkey antianemia factor,[2] these substances were used as supplements to the basal ration. Many groups of rats have been studied, and in each case the animals receiving milk or liver supplements grew at a faster rate than those on the unsupplemented ration, but the differences in rate of gain were never large. Typical results are given in Table I, Groups 2 and 3. Lyophilized whole liver at a level of 3% in the diet gave the best response (Group 4). Certain samples of Wilson's 1:20 liver powder have produced some response (Group 5).

When the response was calculated at different weekly intervals, it was found that in practically all cases the greatest difference was observed at the end of 2 weeks. The results for a few groups calculated at the end of this period are given in Table I (Groups 6 through 10). In this instance, the basal ration was supplemented with additional vitamins and cystine in order to eliminate any possible deficiency of known factors. Under these conditions, the differences were still small but the response to each of the supplements was approximately the same as on the original basal ration.

In an earlier report, Jaffé[5] found that an alcoholic extract of fresh liver produced a growth response in rats fed a natural diet. Therefore, the following ration was fed in order to compare the growth response with that obtained with the synthetic ration: whole ground yellow corn 46.35%, commercial soybean meal 46.35%, corn oil (Mazola) 5%, cystine 0.3%, $CaHPO_4$ 0.92%, $CaCO_3$ 0.6%, NaCl iodized 0.44%, $MnSO_4 \cdot 4H_2O$ 0.04%. Vitamins were added at the same levels as in the synthetic ration. The growth on this unsupplemented diet was greater than on the synthetic basal, but the differences between the basal and the supplemented diets were much more consistent. (Table I, Groups 11 through 15).

[5] Jaffé, W. G., *J. Biol. Chem.*, 1946, **165**, 387.

TABLE II.
Results Obtained with *S. faecalis* Assay.

	Readings on Evelyn	
	Series I units	Series II units
Basal medium	72	70
" " + livers of rats fed basal ration alone	53	52
" " + " " " " " " " + milk	30	43
" " + " " " " " " " + liver	30	46
" " + urine of " " " " alone		45
" " + " " " " " " " + milk		36
" " + " " " " " " " + liver		37

For details of assay see reference 3.

The values noted represent liver samples added at a level of 90 mg (dry weight) per 10 cc of medium, while the urine represents undiluted samples of 1 cc per 10 cc of medium.

The hemoglobin concentration of the blood was determined after the rats had been on the various rations for 4 and 10 weeks. At both periods, the average figures for animals receiving the supplemented and unsupplemented rations were about the same. Differential leucocyte counts obtained after these rats were on experiment for 10 weeks showed no significant variation from the normal.

At the end of 10 weeks, rats receiving diets similar to those used for Groups 1, 2 and 3 were sacrificed and the livers removed for assay with *S. faecalis*.[3] The readings obtained in the Evelyn colorimeter after a 12-hour incubation period are shown in Table II. The results indicate that there is a substance in the liver of rats which stimulates the growth of *S. faecalis*. However, the livers of those animals that had received milk or liver were much more active in stimulating the growth of this organism than those from rats not receiving milk or liver.

In another series, 24-hour urine samples were collected during the ninth week in addition to removal of the livers at the end of this period. The urine from the rats on the basal group produced some stimulation in growth of *S. faecalis*, but the urine of rats fed milk or liver supplements gave greater stimulation (Table II). When this work was repeated with rats receiving the corn-soybean ration, similar results were obtained.

Lactation Studies. Female weanling rats (Sprague-Dawley) were placed on the synthetic ration and supplemented as in Groups 1, 2 and 3. A single dose of vitamin E (10 mg) was administered by dropper to each rat immediately before each mating. The rats were kept in separate cages until the end of the 10th week of experiment, then each group was placed in a large cage. Females were mated with males from our stock colony at the end of the 13th week. After pregnancy was ascertained by a significant weight increase, the female rats were placed in smaller individual wire cages without false bottoms. One day after parturition all litters were reduced to six. The percentage survival was calculated on the basis of the number alive at the end of the 21-day suckling period as compared to the original number at one day of age.

The original experiment consisted of 6 females in a group, and these were remated after a 3-week rest period. The results obtained are presented as the total of both matings.

Ration	% survival
Basal	73
" + 0.5 g fresh liver/day	95
" + 5 cc raw milk/day	66

Similar series have been carried through with approximately the same results. In every case good reproduction has been obtained but the animals receiving the liver have reared a larger percentage of the young left with the mothers.

Discussion. Although a positive response in growth was obtained whenever a liver preparation was added to the synthetic ration, it is evident that the differences in most cases are so small that it is difficult to use this response as an assay for a new factor in liver. Apparently the supply of at least

one additional factor is limiting when rats are fed the basal ration containing 10 B vitamins, but the degree of deficiency seems to decrease as the animals are continued on this regimen. This type of response suggests that the limiting factor is produced within the body, and that the amount produced is not sufficient to meet the needs of the animal during the first few weeks on the ration.

The most consistent differences were obtained with the mixed diet, and it appears that such a diet is most useful for assay purposes. This may be explained by the fact that the intestinal flora supported by a natural ration differs from that which exists on a synthetic ration. The corn and soymeal is not specific in this respect since other natural rations have given similar responses. Geyer *et al.*[6] have noted greater differences between rats fed a synthetic basal and those supplemented with liver, when the animals were kept in tube cages. Better differences may be obtained when coprophagy is prevented in rats fed the natural ration. Cary *et al.*[7] have reported that extra extraction of casein removes a factor "X" necessary for optimum growth of the rat. This may explain the inability to produce significant differences on synthetic rations containing casein which has not been exhaustively extracted with alcohol.

Previous reports showed that Wilson's 1:20 liver powder was inactive for the stimulation of growth of *S. faecalis;* however the samples used in this work were active for both the rat and *S. faecalis*. Thus different samples vary and the activity is similar for the rat and the microorganism. When the activity of liver or urine from rats fed the basal is compared to that of rats fed the active material, there is always an increased activity in the case of supplemented rats. This change suggests an increased storage of the factor in the liver, as well as increased excretion when a dietary source is fed. Previous work[8] has demonstrated clearly that the B_C potency of the liver was increased when the dietary intake of folic acid was increased.

The fact that the urine and liver of rats fed the unsupplemented ration stimulates growth of *S. faecalis* may be due to the production of the factor by the intestinal flora, to the presence of an undetectable amount of the factor in the basal ration, or to the storage of the factor in the animal body during the pre-experimental period. It is also possible that *S. faecalis* may be stimulated by more than one factor. This last possibility is being investigated.

The results obtained in the lactation studies are in general agreement with reports from other laboratories. Richardson and Hogan[9] reported improved lactation in rats upon addition of a fullers earth eluate of liver, which supplies B_C and probably unrecognized vitamins. A liver filtrate which contained a low level of Vitamin B_C was just as active in this respect. Cerecedo and Vinson[10] reported a beneficial effect on lactation when a folic acid concentrate was added to the diet. It is quite possible that these concentrates are sources of unknown nutrients essential for maximum lactation in the rat. Spitzer *et al.*[11] obtained better lactation when Wilson's 1:20 liver powder was added to a mixed diet which was unsatisfactory for lactation.

In the series reported in this paper, folic acid and biotin were used in crystalline form. The fact that a significant increase in percentage survival of young is attained when fresh liver is added to this diet, indicates that the liver contains a factor(s) necessary for maximum survival of young. It is quite possible that the same factor is concerned in the growth stimulation and in the improved lactation.

6 Geyer, R. P., Geyer, B. R., Derse, P. H., Zinkin, T., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, 1947, **33**, 129.

7 Cary, C. A., Hartman, A. M., Dryden, L. P., and Likely, G. D., *Fed. Proc.*, 1946, **5**, 128.

8 Schweigert, B. S., Teply, L. J., Greenhut, I. T., and Elvehjem, C. A., *Am. J. Physiol.*, 1945, **144**, 74.

9 Richardson, L. R., and Hogan, A. G., *Fed. Proc.*, 1945, **4**, 161.

10 Cerecedo, L. R., and Vinson, L. J., *Arch. Biochem.*, 1944, **5**, 469.

11 Spitzer, R. R., and Phillips, P. H., *J. Nutr.*, 1946, **32**, 631.

Summary. Although rats grow at a rapid rate on a synthetic diet, it is possible to obtain small increases in the rate of growth by the addition of liver and liver preparations. The differences can also be obtained when the basal ration contains adequate amounts of folic acid. A larger and more consistent response to liver can be demonstrated when a corn-soybean meal ration is used. The addition of liver to the synthetic ration fed female rats increased the percentage of young surviving during the lactation period.

A factor in liver has been shown to stimulate the growth of *S. faecalis*. Assay with this organism indicates that an increased amount of the factor is excreted in the urine and stored in the liver of rats fed liver preparations. It is suggested that more rigorous methods are needed, such as prevention of coprophagy, use of natural rations or more extensive treatment of the components of the synthetic ration in order to devise suitable assay procedures.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins; to the Wilson Laboratories, Chicago, Ill., for liver powder 1:20 and whole liver powder; to E. R. Squibb and Sons, New York, for desiccated whole liver and lyophilized liver; to Lederle Laboratories, Inc., Pearl River, N. Y., for synthetic folic acid and several liver fractions.

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Some Pharmacological Characteristics of Bacitracin II. Absorption and Excretion of Bacitracin in the Dog.*

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Continuing an investigation of the pharmacological characteristics of bacitracin,[1] we have studied the absorption and excretion of the antibiotic in the dog. Because such studies are of fundamental importance in the rational use of the drug, the following data are presented.

Experimental. One female and 5 male mongrel dogs, weighing 7.5 to 11 kg, maintained on a stock diet and water *ad libitum,* were fasted over-night before use. In general, fasting dogs were given 200 cc of water by stomach tube at 9:00 a. m. and again at noon to insure an adequate diuresis. The antibiotic, a single lot designated B-100,† was administered by various routes at approximately 9:30 each morning, and sterile blood samples were taken at specified time intervals thereafter. The blood was permitted to clot and sterile samples of serum, diluted one to 3, were used directly in the assay procedure. Urine samples were collected 7 and 24 hours after administration of the drug. Occasionally 3- and 5-hour urine samples were collected in connection with distribution studies. These, and the routine 7-hour samples were taken by catheter. The 7- to 24-hour specimen was collected in a metabolism cage as a total sample. The urine samples were diluted one to 10 and passed through a Selas filter before testing. For stool analysis, a 1 g aliquot of the well mixed specimen was triturated with 50 cc of water and the clear supernatant fluid was passed through a Selas filter before testing.

The test procedure, involving the inhibition of the Chanin strain of β-hemolytic strep-

* The work described in this paper was done under a contract between the Office of the Surgeon General and Columbia University. Administration of the contract was directed by Dr. Frank L. Meleney.

1 Scudi, J. V., and Antopol, W., PROC. SOC. EXP. BIO. AND MED., 1946, **64**, 503.

† We are indebted to Dr. John T. Goorley of the Ben Venue Laboratories for the bacitracin.

tococci, Group A, was designed by Miss Balbina Johnson, and will be described by her elsewhere. In our hands, the method gave sharp end-points at .001 ± .0003 units of bacitracin per cc. In a series of 29 analyses of standard solutions containing 3.0 units of bacitracin per cc, the mean value was 2.94 ($\sigma = 0.5$). Control urine and stool filtrates gave no inhibition of the organism in the test procedure, and recovery of added bacitracin‖ was satisfactory in both cases. Recovery of bacitracin added to serum, however, was not good. In a series of 38 experiments involving bacitracin added in concentrations ranging from .03 to 1.0 units per cc of serum, the mean recovery was 42% ($\sigma = 11.3$). All serum values were, therefore, corrected by dividing by 0.42. The data submitted in the following pages are subject to these wide variations, but no other analytical method is yet available. The greatest variations occurred in attempting to duplicate analyses on different days. To keep such variations minimal and to obtain at least comparable data, all analyses in each experiment were performed in duplicate on the same day whenever possible.

Results. Following oral administration in doses of 3,000 and 6,000 units per kg body weight, no detectable bacitracin (that is, less than 0.01 unit per cc of serum) was found in the blood stream, nor was any found in the urine during the 24 hours after administration of the drug. Following oral administration of 1,500 units per kg body weight to 2 dogs, less than 5% of the oral dose was recovered in the stool. Consequently, it appears that the antibiotic is largely destroyed in the gastro-intestinal tract. This finding is in agreement with the remarkable lack of oral toxicity reported in the mouse.[1] The presence of small but significant amounts of bacitracin in the stool suggests that the drug may be useful in the treatment of infections of the intestinal tract. Subcutaneous injection of the larger doses gave the blood concentrations of bacitracin shown in Fig. 1. It is of interest to note that appreciable concentrations remain in the blood as long as 7 or 8 hours after a single injection of the drug. Peak concentrations are relatively low compared to those observed following intramuscular and intravenous injection. This observation suggests, as an explanation for the low acute subcutaneous toxicity in the mouse, (1) that the balance of

‖ These were simple recovery experiments and did not include an aging study with reference to possible inactivation of the antibiotic.

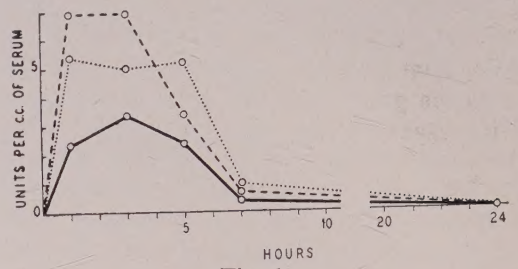

Fig. 1.
Serum concentrations following subcutaneous injection of 6000 u/kg (dot-dash lines) and 3000 u/kg (solid line) of bacitracin.

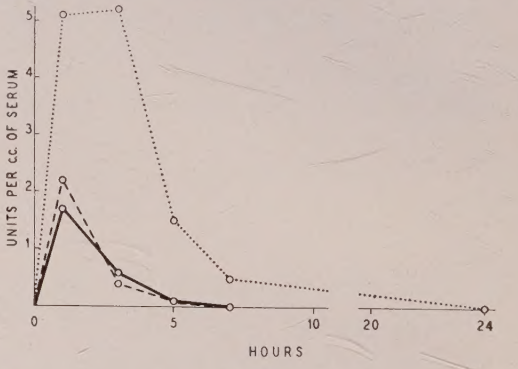

Fig. 2.
Serum concentrations following intramuscular injection of 3000 u/kg (dotted lines) and 1000 u/kg (dashed and solid lines) body weight of dog.

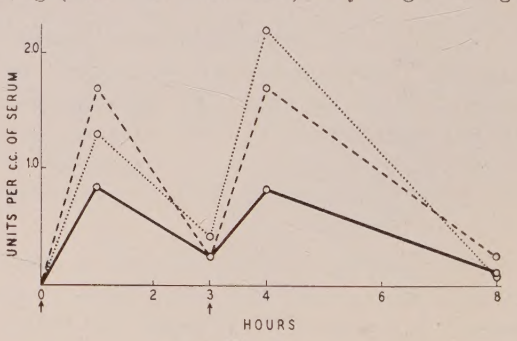

Fig. 3.
Serum concentrations following intramuscular injections of 1000 u/kg in each of three dogs. Arrows indicate times of injection.

absorption, detoxication and excretion of the drug, which keeps the blood levels low, prevents the accumulation of lethal concentrations at higher centers. Following intramuscular injections of 1,000 and 3,000 units per kg body weight, relatively higher blood concentrations were observed (Fig. 2). These were not quite so persistent as those following subcutaneous injection, but at the higher dose level an appreciable concentration remained 7 hours after dosing. Repeated intramuscular injections of 1,000 units per kg gave the data shown in Fig. 3.

None of the dogs exhibited toxic signs in the course of the preceding experiments, but following the rapid intravenous injection of concentrated solutions central effects were noted. Injection of 3,000 units per cc of solution at the rate of 3 to 4 cc per minute until a dose of 3,000 units per kg had been given, caused in order of appearance, salivation and associated signs of nausea, spastic, outstretched hind quarters, and a scoliosis owing to muscle spasm. These signs disappeared within 5 minutes after the drug was administered. With another dog given 1,000 units per kg within one minute (concentration 1,000 units per cc) evidence of nausea and muscle spasm appeared. On the other hand, when 3,000 units per kg were given over a 6-minute period in the form of a solution containing 1,000 units per cc, the only signs were those of nausea and a slight central depression, both of which disappeared rapidly. Lower doses given more slowly produced no noticeable effects. These observations suggest that at the present stage of its purification, bacitracin should be administered slowly and in dilute solution, if the drug is given intravenously. Indeed, persistence data suggest that subcutaneous or intramuscular administration may be the routes of choice. The data obtained following intravenous administration are shown in Fig. 4. Here, the blood concentration—time curves rise and fall precipitously. At a dose level of 300 units per kg body weight, a maximum of 3.6 units per cc is attained and the blood is essentially cleared of the drug in 4 hours. At higher dose levels the maxima and the persistence are increased.

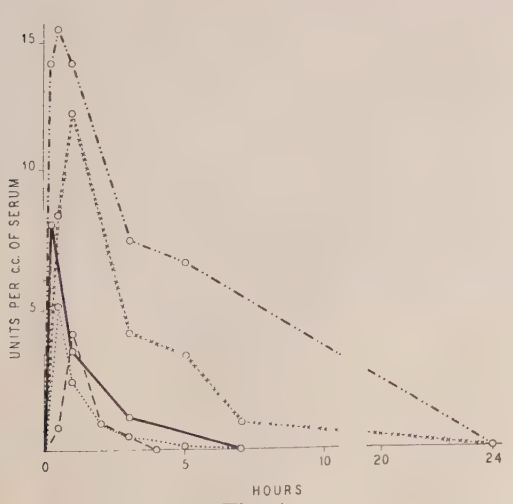

Fig. 4.
Serum concentrations following intravenous injection of 3000 u/kg (dot-dash and cross-hatched lines) 1000 u/kg (solid and dotted lines) and 300 u/kg (dashed lines),

The urinary excretion data obtained in conjunction with these experiments are presented in Table I. The lack of urinary excretion of the drug following oral administration has been considered. Following other modes of administration, the recovery of bacitracin appears, in general, to increase with the dose administered, but the recoveries are widely variable. As little as 7 or as much as 98% of the dose may be recovered in the urine. Relatively high concentrations persist in the urine for more than 7 hours after a single dose of the drug, and significant concentrations are found in the 24-hour specimen. This persistence in the urine may make the drug useful in urologic conditions.

In a study of the distribution of bacitracin between erythrocytes and plasma, 3,000 units of the antibiotic per kg body weight were injected intravenously, and after one hour, sterile blood samples were collected under oil. The samples were defibrinated and centrifuged anaerobically. Quadruplicate analyses, performed on the whole blood, serum and erythrocytes‡ gave the following average values: 8, 14 and less than 0.2 units per cc, respectively. The whole blood concentration of 8 units per cc was in good agreement with the value of 8.4 units per cc calculated from

‡ Bacitracin concentrations as high as 100 units per cc did not alter the fragility of the erythrocytes.

TABLE I.
Urinary Excretion of Bacitracin in the Dog.

Mode of administration	Dose given Units/kg	Total	7-hr urine Units/cc	Total	24-hr urine Units/cc	Total	Total	% recovery
Oral	6000	38,400	0	0	0	0	0	0
"	3000	18,900	0	0	0	0	0	0
Subcutaneous	3000	33,000	20	9,700	8	2,100	11,800	36
"	6000	63,000	35	15,500	4	1,600	17,100	27
"	6000	43,200	—	—	32	23,600	23,600	55
Intramusc.	1000	9,200	18	6,500	1	590	7,100	77
"	1000	7,400	14	1,800	2.3	400	2,200	30
"	3000	27,300	92	26,000	3	1,100	27,100	98
Intravenously	300	3,300	0.4	200	0.2	50	250	7
"	1000	7,200	—	—	3	1,300	1,300	18
"	1000	7,300	56	2,000	1.3	100	2,100	29
"	3000	21,000	0.6	300	2.3	8,900	9,200	44
"	3000	18,600	72.0	4,300	9.0	3,600	7,900	42

the serum concentration. These findings are in agreement with results obtained by adding bacitracin to whole blood, cells, and serum *in vitro*. Two types of experiments were performed. In the first, 6.66 units of bacitracin were added per cc of serum.§ The average recovery in triplicate experiments was 5.5 units per cc. The original red blood cells were then added to the serum and the reconstituted blood was incubated for 10, 30 and 60 minutes, and the serum was separated. Triplicate analyses of the serum gave an average value of 5.2 units per cc. Thus, none of the bacitracin was taken up by the cells. As an additional control, 5 units of bacitracin was added per cc of red cells. Following hemolysis triplicate analyses gave an average recovery of 5.1 units of bacitracin per cc of cells. In the second type of experiment, bacitracin was added to whole blood and the system was incubated for 30, 60 and 120 minutes. In one series of experiments, 2 units of bacitracin were added per cc of whole blood. The average recovery from serum in 6 analyses was 2.2 units per cc, and the cells contained less than 0.1 unit per cc. In another series of experiments, 6 units of bacitracin were added per cc of whole blood, and again the system was incubated for the same periods of time. The average recovery from serum in 3 determinations was 5.2 units per cc, and the red blood cells contained less than 0.2 unit per cc. Thus, it may be concluded that bacitracin does not penetrate the red blood cell. In connection with these findings, it was of interest to calculate the *approximate* volume of distribution of the drug in the dog. In 4 analyses, the average volume of distribution after intravenous or intramuscular injection was 40% of the body weight as shown in Table II.

That bacitracin is not freely diffusible in the animal organism was further demonstrated by comparison of the concentrations in the serum and spinal fluid of the monkey. In a series of 5 monkeys, used in chronic toxicity studies which will be reported later, serum concentrations of 6.6, 0.75, 0.18, 8.1 and 6.6 units per cc were found. Analysis of spinal fluid taken at the same time gave concentrations of 0.34, .009, .08, .34 and .34 unit per cc of spinal fluid, respectively.

Summary. The absorption and excretion of bacitracin have been studied in the dog. Only small fractions of the oral dose were recovered in the stool. Since none was found in the blood or urine after oral administration and little was found in the feces, it appears that the antibiotic was largely destroyed in the gastro-intestinal tract. Following parenteral administration of large single doses, significant concentrations of the drug persisted in the blood stream for as long as 7 or 8 hours. Other than slight and transient central effects produced by excessively rapid intravenous injection, the animals showed no apparent signs of toxicity in spite of the fact that doses as high as 6,000 units per kg body

§ Recoveries of added bacitracin at these higher concentrations was uniformly better than 42%. The data in this section therefore are given directly without any correction factor.

TABLE II.
Apparent Volume of Distribution* of Bacitracin in Male Mongrel Dogs.

Dog No.	Wt kg	Total dose in units	No. hr after inj.	Plasma conc. units/cc	Total urinary output in units	Apparent volume of distribution in liters	% body wt
156	6.4	19,200†	3	7.5	2,200	2.3	36
156	6.4	19,200†	5	6.7	4,300	2.2	35
151	7.3	7,300†	3	1.3	2,000	4.1	56
229	9.2	27,600‡	7	0.44	26,200	3.2	34

* The total dose, minus the fraction excreted, divided by the plasma concentration gives the number of liters of body water in which the bacitracin appears to be distributed. The apparent volume of distribution is expressed in terms of body weight.
† Dose administered intravenously.
‡ " " intramuscularly.

weight were administered. The recovery of the drug in the urine appeared, in general, to increase in direct ratio to the dose administered, but the recoveries were widely variable. Significant concentrations of bacitracin persisted in the urine for more than 7 hours after administration of a single dose of the antibiotic. Bacitracin is not freely diffusible. It did not penetrate the red blood cell, nor did it enter the spinal fluid freely.

15847 P

Effect of Muscular Activity on Curarization in Rabbits.

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In studying the curariform action of a group of quaternary quinine derivatives by the rabbit head-drop method, one of us[1] observed that if rabbits struggled following a single intravenous injection of these paralyzing substances, head-drop seemed to occur more rapidly than usual. More recently, in the course of studies with *d*-tubocurarine, it was observed that shortly after recovery from, or even during, convulsions induced in rabbits by the injection of cocaine or veratrine, the amount of *d*-tubocurarine required to produce "head-drop" was markedly reduced. Torda and Wolff[2] have demonstrated the presence of a curare-like agent in the serum of blood obtained from the hind limbs of anesthetized cats following tetanic stimulation of the sciatic nerve or following passive exercise of the extremities. In view of these findings, quantitative studies have been made of the synergistic effect of exercise on curarization, using the rabbit "head-drop" assay method.

Method. Several types of muscular exertion were investigated.

(1) *Running.* Rabbits were exercised in a long corridor for 5 minutes, in response to gentle stimuli from the experimenter, who followed the animal at a rate no faster than a normal walk. Animals, thus exercised, showed signs of exertion such as increased respiration, but, of course, were by no means exhausted. This method proved more satisfactory than were attempts to exercise rabbits in a treadmill.

(2) *Violent exertion.* When the hind legs of rabbits were grasped and held off the floor, the animals made violently active attempts to escape; these repetitive efforts were punctuated by brief intervals of rest. One-half-

1 Chase, H. F., Lehman, A. J., and Rickards, E. E., *J. Pharm. and Exp. Therap.*, 1944, **82**, 266.
2 Torda, C., and Wolff, H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 242.

TABLE I.
Rabbit Head-Drop Doses (mg per kg) of *d*-Tubocurarine Before and After Exercise.

	Type of exercise			
	5 min running	½ min violent	2 min violent	10 min hind legs
	(30)*	(8)	(10)	(10)
Before exercise	0.245	0.252	0.268	0.258
After "	0.112	0.16	0.119	0.151
Critical ratio	16.3	5.4	10.0	5.4
% reduction	54%	36%	55%	41%

* No. of rabbits.

and 2-minute periods of this extreme type of muscular exertion were investigated.

(3) *Moderate exercise of hind limbs only.* Rabbits were placed in their usual sitting posture and gently restrained manually. The hind legs were pulled alternately from under the animal at a rate of 100 to 120 times per minute for a period of 10 minutes. During the latter part of the period passive exercise was often necessary, though even then some voluntary resistance was noticeable. If the animal moved forelimbs or body it was momentarily released and such efforts promptly ceased.

Within one minute after exercise the "head-drop" assay was started. Increments of 0.05 cc of a solution of *d*-tubocurarine containing 0.65 mg (4.0 "Intocostrin" units) per cc were administered intravenously every 15 seconds until head-drop occurred. Control and test values were obtained on the same animals by the cross-over technic.

Results. The data in the accompanying table demonstrate that in the production of head-drop in rabbits there is a definite synergism between *d*-tubocurarine and muscular activity. The physiological significance of these results is indicated by the fact that they are demonstrable after only one-half minute of intermittent, but strenuous exercise; further, a prolongation of the period of exertion to 2 minutes measurably augmented the synergism. These findings would tend to substantiate the findings of Torda and Wolff and of others.

Comment. The mechanism of this apparent synergism is being investigated further. Possibly a central synaptic fatigue of postural reflexes is involved, exhaustion of neurohumoral mechanisms at the nerve endings might occur, or an accumulation of products of muscle metabolism may be involved.

The demonstration of this susceptibility of overactivated muscles to the action of curarizing agents may serve to explain the clinical observation that the hyperinnervated muscle groups of spastic paralytics respond to doses of curare which have no demonstrable influence on normally innervated muscles. In anesthesia these findings suggest greater exercise of caution in the use of curare following a stormy induction, in which the patient is hyperactive, than following a quiet induction.

This phenomenon may help in explaining the beneficial action of sodium amytal which, by preventing convulsions, protected rats against ordinarily fatal doses of *d*-tubocurarine.[3]

Summary. Muscular exercise has been demonstrated to be synergistic with *d*-tubocurarine in producing head-drop in rabbits. Synergistic effects are apparent following short bouts of exercising and the degree of additive effect increases with the duration and intensity of the exercise.

[3] Cohnberg, R. E., *J. Lab. and Clin. Med.*, 1946, **31**, 866.

15848

Absorption of Penicillin Through the Human Vagina.

Melvin Schudmak and H. Close Hesseltine.

*From the Department of Obstetrics and Gynecology, The University of Chicago and The Chicago Lying-in Hospital.**

Several routes of administration for penicillin have been thoroughly evaluated. While the investigation of the value of penicillin in cocoa base suppositories for vaginal infections was underway 2 reports by Roch, Barker, and Bacon[1] and Goldberger, Walter and Lapid[2] indicated somewhat comparable absorption rates. According to our results the absorption of penicillin from the vagina was not as reliable, regular, nor as complete as indicated by these 2 groups of workers.

In preliminary tests 100,000 units of penicillin in a single cocoa base suppository was inserted into the vagina approximately 12 hours before the collection of blood for the determination of blood level. Because of the consistently low levels tests were made in a subsequent series within 4 hours and the dosage was increased to 200,000 units twice daily with a few exceptions. As these were all out-patient cases the blood samples were collected thereafter about the third hour. The level of penicillin in the blood was determined by the test tube dilution method of Kolmer.[3] The tests were run in duplicate except when there was insufficient serum. The Oxford strain *Staphylococcus aureus H.* was used as the test organism. Tests with known dilutions were made at the same time to assure stability of the test bacteria.

The patients were all under treatment for vaginal trichomoniasis except patient 14. This patient had an undetermined type of vaginitis. The patients were supplied with cocoa base vaginal suppositories each containing 100,000 units. These suppositories were stored at ordinary refrigerator temperature. The patients were instructed thoroughly on the storage and care of the medication. Four patients, 2 pregnant and 2 nonpregnant received but 100,000 units daily, whereas the remaining 12 had 200,000 units applied twice daily. The duration of the gestation varied from 6 to 31 weeks. There was no difference noted in one patient between the 13th and 24th weeks. In the nonpregnant, only one patient, 13, revealed any marked difference in blood levels for different phases of the menstrual cycle.

Table I lists the details.

Complete bacteriologic studies were not undertaken during this survey period. However the routine smears did not reveal a conversion to the normal type I vaginal flora. No attempt has been made to correlate inhibitory action of bacteria in the vagina upon the penicillin.

Six (3 pregnant, 3 nonpregnant) patients did have symptomatic and clinical improvement but in not one instance was a cure accomplished. No additional therapy as douches was allowed. Thirty tests were completed. The highest readings were 1 unit once, 0.256 unit twice and 0.128 unit once.

Conclusions. According to the condition of this experiment cocoa base suppositories containing penicillin were not a satisfactory procedure for the treatment of vaginal trichomoniasis.

The blood levels attained under the condition of the experiment, would have been

* Supported in part by Chicago Lying-in 50th Anniversary Fund for Research on Puerperal Infection.

1 Rock, J., Baker, R. H., and Bacon, W. B., *Science*, 1947, **105**, 13.

2 Goldberger, M. A., Walter, R. I., and Lapid, L. I., *Am. J. Obst. and Gynec.*, 1947, **53**, 529.

3 Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic, 4th Edition*, Appleton Century, New York, 1945.

TABLE I.
Absorption of Penicillin from Cocoa Base Suppositories Through the Human Vaginal Mucosa. All but One of these Patients Had Vaginal Trichomoniasis.

Patient No.	Age	Weeks pregnant	Penicillin Units	Penicillin Time in vagina	Clinical result	Blood level
			Obstetric Patients.			
			× 100,000			
1.	26	6	2	4	improved	.032
2.	21	12	1	3	”	.032
3.	23	13	1	3	unimproved	.008
		24		2		.008
4.	26	31	2	3	improved	.128
			Gynecologic Patients.			
		Day of cycle				
5.	25	Postpartum 3 mo.	2	3	failure	.008
6.	28	6	2	2	improved	1.
7.	33	21	2	3	failure	.008
8.	29	28	1	2	improved	.256
9.	37	28	2	3	failure	.008
10.	23	7	2	3	improved	.008
		14		3		.016
		21		3		.008
11.	30	12	2	4	unimproved	.008
		19		3		.016
		26		3		.016
		5		3		.016
12.	33	24	2	4	”	.008
		3		4		.008
		10		4		.016
		17		2		.008
13.	37	27	2	3	failure	.032
		6		4		.008
		13		3		.256
		20		3		.008
14.	25		1	2	improved	.064
15.	30		2	3	unimproved	.008
16.	30	Surgical menopause	2	3	failure	.008
				3		.008
				4		.008

inadequate therapeutically in most instances. The rate of penicillin absorption through the vaginal mucosa is unpredictable. Therefore penicillin should be used in the vagina only for local conditions but these conditions have not yet been established.

We thank the Schenley Laboratories for their generous supply of penicillin suppositories.

15849 P

Streptomycin in Experimental Ocular Infections.*

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Leopold and Nichols[1] have demonstrated that streptomycin administered to rabbits in dosages of 10,000 μg per kg body weight appears in the conjunctiva, sclera, extraocular muscles and aqueous humor. Much larger doses were necessary to obtain detectable amounts in the posterior segment of the eye. The intact cornea is not penetrated. Following abrasion, or by means of iontophoresis, streptomycin is found in the aqueous humor.

In the experiments to be described, rabbits were anesthetized by intravenous injection of nembutal. The streptomycin† of desired strength was applied as a saline solution, except where noted as the dry powder.

This paper reports the following observations:

1. Factors increasing penetrability through the cornea: We confirmed Leopold and Nichols'[1] observation that streptomycin does not penetrate the normal cornea, but does following abrasion and upon iontophoresis.

In our experiments with the abraded cornea, 100 μg per ml aqueous were obtained when a constant corneal bath of 10,000 μg streptomycin was applied for 2 hours. Iontophoresis of the unabraded cornea (22 v., 2 ma., anode to solution cup) for 30 minutes with a solution of the same concentration gave 25 μg per ml aqueous.

Increased penetrability occurred in the inflamed cornea. Values of between 25 and 200 μg per ml aqueous were obtained in 15 to 60 minutes following application of a bath containing 10,000 μg streptomycin per ml. This variation is related more to the size of the lesion than to duration of application.

In the intact eye, the addition to the corneal bath of one drop of 0.5% Areosol O.T. per ml of streptomycin solution resulted in increased penetration simulating values obtained upon abrasion. With a solution containing 10,000 μg of streptomycin per ml, 25 μg per ml of aqueous were obtained in 2 hours; with a bath concentration of 50,000 μg, values of 25 to 50 μg were obtained in 30 to 60 minutes. If 10 drops Areosol solution were added per ml of streptomycin solution, values of over 100 μg were obtained, with definite damage resulting to the cornea.

2. Local toxic effect upon application to the eyeball: In the abraded corneas as established by fluorescein staining, 3 instillations per day of a streptomycin solution containing 10,000 μg per ml produced no delay in healing, whereas a solution containing 50,000 μg per ml, or the use of the dry powdered streptomycin caused a definite retardation of healing to at least twice the normal period. With the higher concentrations, scar formation and vascularization also occurred.

3. Effect of intraocular injection: Direct injection of 0.1 ml streptomycin saline solution in concentrations varying from 250 to 10,000 μg per ml into the center of the vitreous humor produced negligible opacities when observed by means of the ophthalmoscope and slit-lamp. Inoculation by injection of 0.1 ml of a 24-hour broth culture of a virulent strain of *Str. pyogenes* simultaneously with the streptomycin solution resulted in complete protection against infection. This was true if the streptomycin was administered intravitreally up to 6 to 8 hours following inoculation. The control infected eye progressed to eventual abscess formation.

* This research was done under a contract between the Navy Program for Basic Research and Northwestern University.

1 Leopold, I. H., and Nichols, A., *Arch. Ophth.*, 1946, **35**, 33.

† We are indebted to Dr. Donald Robertson, Associate Medical Director, Merck & Co., Inc., Rahway, N.J., for the streptomycin used in this investigation.

4. Treatment of experimental *B. pyocyaneus* infections of the cornea: Ulcers of uniform severity were produced by inoculation of the cornea with a 24-hour broth culture of a virulent strain of *B. pyocyaneus*. Treatment consisted of 3 applications (drops) at 2-hour intervals of streptomycin solution containing 10,000 μg per ml. This form of treatment afforded complete protection when commenced within 6 hours following inoculation. The infected control eyes uniformly progressed to complete destruction of the cornea.

Summary. 1. The penetrability of streptomycin through the cornea may be increased by abrasion, inflammation, ion-transfer and wetting agents. 2. No local toxic effects were noted when saline solutions of streptomycin containing up to 10,000 μg per ml were used. With concentrations of 50,000 μg or as a dry powder, delayed healing occurred. 3. Intraocular injection, in amounts up to 1,000 μg of streptomycin in 0.1 ml saline were well tolerated. Smaller amounts (25 to 300 μg) were therapeutically effective up to 6 to 8 hours against a virulent strain of *Str. pyogenes*, though transient or negligible vitreous opacities occurred with these concentrations. 4. Experimental corneal ulcers produced by injection of *B. pyocyaneus* were prevented when treatment was started within 6 hours after inoculation by 3 applications at 2-hour intervals of a saline solution of streptomycin containing 10,000 μg per ml.

15850

Further Studies on the Mechanism of Alloxan Diabetes, Pancreatectomy and Alloxan.*

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The experiments to be presented here were performed in an attempt to investigate further the significance of the initial blood sugar fluctuations after alloxan and the character of the ensuing diabetes. To be more specific, they concern the problems of the pancreatic origin of the alloxan hypoglycemia and the interrelationship of external and internal pancreatic secretion in the development of alloxan diabetes.

The typical triphasic fluctuation of the blood sugar after a diabetogenic dose of alloxan is well known. An initial hyperglycemia is followed within a few hours by a secondary hypoglycemic phase which may last from 6 to 10 hours and after which the final persistent hyperglycemia and glycosuria develops. It is important to emphasize that the 2 initial phases, especially the secondary hypoglycemia, vary in severity with different species. In the dog for instance these fluctuations are rather mild and asymptomatic, while the rabbit is thrown into severe hypoglycemic shock and convulsions which it survives only if large amounts of glucose are given repeatedly over several hours.

The hyperglycemic phase is absent in adrenalectomized rabbits[1] and depends to a certain degree on the nutritional state of the animals and the glycogen stores of the liver. It is absent, too, in hepatectomized animals.[2] The explanation that it is extrapancreatic in origin[1] has found general confirmation and acceptance.

The secondary hypoglycemia starts at about the same time when histological examination of the pancreas begins to discover degenerative changes in the beta cells as

* This work has been supported by grants from the Douglas Smith Foundation for Medical Research and from the Eli Lilly Research Laboratories, Indianapolis.

[1] Goldner, M. G., and Gomori, G., *Endocrinology*, 1944, **35**, 241.

[2] Houssay, B. A., Orias, O., and Sara, T., *Science*, 1945, **102**, 197.

TABLE I.
Blood Sugar Fluctuation Following the I.V. Injection of a Diabetogenic Dose of Alloxan into Dogs Which Were Pancreatectomized Several Days or Weeks Prior to the Experiment.

Dog N R	Days after pancreatectomy	Insulin treatment	Fasting blood sugar	Dose of alloxan, mg/kg	Blood sugar level (Hr after injection of alloxan) 1	2	3	4	5	6	8	24	48
122	11	+	156	50	151	155	157	175	—	165	—	161	136
214	12	+	168	50	190	194	214	—	218	—	212	198	151
142	7	0	297	50	—	315	—	295	—	254	—	312	278
528	6	0	332	50	329	249	334	324	317	338	—	340	—
186	30	0	288	50	288	321	324	311	—	—	—	304	314
132	6	0	266	60	—	290	—	286	—	266	—	268	306
221	21	0	258	75	269	238	—	208	—	211	—	283	264
168	40	+	219	75	227	238	—	203	—	220	—	247	186
123	6	0	273	100	322	337	—	307	302	—	290	303	244

pyknosis, cloudy swelling and degranulation. On the basis of this observation and others, as for instance that the fall of the blood sugar fails to occur only then when the dose of alloxan is insufficient to induce necrosis of the islet cells, it was suggested that the secondary hypoglycemia is pancreatic in origin and due to stored and preformed insulin leaking out of the degenerating beta cells.[1] This explanation has found confirmation by many workers, but of late has been challenged by others. Particularly Houssay and his co-workers[2,3] have reported that they have observed hypoglycemic reactions in dogs that were pancreatectomized shortly prior to the injection of alloxan. They therefore ascribed the secondary hypoglycemia to an extrapancreatic mechanism and suggested that it may be due to a primary effect of alloxan upon the liver. Since they do not deny the direct effect of alloxan upon the islet cells nor the pancreatic origin of the ensuing diabetes, one would have to conceive that alloxan produces hypoglycemia simultaneously by 2 independent actions upon 2 different target organs, *i.e.* in the pancreas by release of insulin, in the liver by prevention of glycogenolysis. Unlikely as this coincidence may appear theoretically, we felt necessary to review and to extend our own experiments on the effect of alloxan upon depancreatized dogs. We have failed to find any alloxan hypoglycemia in such animals. Our observations were made on 4 different groups of experiments. Table I shows the fluctuation of the blood sugar when a diabetogenic dose of alloxan is given to dogs which had been depancreatized several days or weeks prior to the acute experiment. The first group of dogs whose diabetes was treated adequately with insulin and the second group of dogs with uncontrolled diabetes showed the same negative reaction of their blood sugar, the initial blood sugar level being the only difference. No doubt, the glycogen stores of the livers differ greatly in these 2 groups, yet hypoglycemia did not occur in either one. Similar results were obtained in 2 series of acute experiments (Table II). In the first one, the animals were depancreatized 30 minutes prior to the injection of alloxan. This is the procedure with which the South American workers obtained their positive results. Here too, no hypoglycemia occurred in our animals. The last group are animals in which a functional pancreatectomy was performed. The pancreas was freed and its blood supply interrupted by clamping of all vessels for 5 minutes during and after the injection of alloxan. It has been shown in earlier experiments that alloxan is rendered innocuous to the islet cells within 5 minutes after injection.[4] Thus any fluctuation of the blood sugar after alloxan, observed in these animals, must be extrapancreatic in origin—if the clamping of the vessels is complete. All 4 animals showed a moderate initial hyperglycemia and no hy-

[3] Houssay, B. A., Orias, O., and Sara, T., *Rev. de la Soc. Arg. de Biologia*, 1945, **21**, 30.

[4] Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 232.

TABLE II.

Fluctuation of Blood Sugar Following I.V. Injection of Diabetogenic Dose of Alloxan in Dogs, the Pancreas of Which Had Been Either Extirpated 30 Days Prior to the Experiment or Clamped for 5 Days After the Injection of Alloxan.

Dog N R	Blood sugar fasting	Type of operation	Blood sugar after operation	Dose of alloxan, mg/kg	Blood sugar level (Hr after injection of alloxan) 1	2	3	4	5	6	8	10	24	48
409	108	Total pancre-	176	60	192	196	272	276	216	—	252	—	332	356
94	106	atectomy	148	60	202	228	280	322	328	—	312	240	284	296
188	72		104	60	98	107	110	124	121	—	152	—	186	318
478	94		89	60	116	140	154	194	166	—	160	—	276	—
165	78	Pancreas clamped	—	60	100	74	72	75	80	73	—	—	84	112
503	96	for 5 min.	—	60	186	116	102	70	61	60	68	—	70	188*
88	90	after inj. of	—	60	144	125	113	89	115	99	101	—	116	104
141	94	alloxan	—	75	122	134	120	94	90	110	112	100	96	96

* This dog developed diabetes. Histological examination revealed that the clamping had been incomplete.

TABLE III.

Blood Sugar Fluctuation Following the I.V. Injection of a Diabetogenic Dose of Alloxan in Rabbits After Partial Pancreatectomy.

Rabbit N R	Time after partial pancreatectomy	Dose of alloxan, mg/kg	Blood sugar level (Before and hours after injection of alloxan) 0	1	2	4	6	8	10	24	48
	Days										
29	12	200	110	232	280	262	120	70	—	180	304
43	10	200	75	184	299	236	82	66	72	195	258
44	21	200	90	210	326	278	56	58	80	212	336
56	21	200	106	170	257	302	124	60	—	198	282
	Min.										
62	30	200	94	144	196	165	102	80	—	182	254
63	30	200	103	175	244	254	148	63	—	206	290
75	30	200	89	160	220	208	94	70	—	240	312
22	Intact control	—	113	232	304	82	47	31*	—	—	—

* Died in hypoglycemic convulsions.

poglycemia occurred in 3 of these animals—none of which developed diabetes. Only one dog, NR. 503, showed a secondary hypoglycemia, but also developed diabetes. Biopsies from the pancreas of this dog showed typical islet cell degeneration and it must be concluded that here the interruption of the blood supply was incomplete. If the hypoglycemia was due to hepatic dysfunction it should not have been followed by diabetes and it should have occurred in the 3 others which did not develop diabetes.

It has been mentioned above that the secondary hypoglycemia is much more marked in rabbits than in dogs. It seemed, therefore, desirable to repeat these experiments in this species. Unfortunately, total pancreatectomy in rabbits is an almost impossible procedure, in a one-stage operation. A 2-stage operation, on the other hand, precludes acute experiments. We therefore have confined ourselves to observations on partially depancreatized rabbits. The operation was performed under sodium pentothal and the splenic part of the pancreas was removed as far as possible. The part close to the portal vein and the duodenum remained *in situ*. Controls with anesthetized rabbits which were not operated upon showed that anesthesia did not modify significantly the blood sugar fluctuation. Table III represents the values of 3 acute and 4 chronic experiments as compared with the reaction of the intact control animal and demonstrates that partially depancreatized rabbits develop after alloxan a less severe hypoglycemia than intact animals.

TABLE IV.
Ligation of Part of the Pancreatic Duct Does Not Protect the Islets in the Ligated Part of the Pancreas Against the Degenerative Effect of Alloxan.

Dog N R	Days after partial ligation of pancreatic duct	Dose of alloxan, mg/kg	Blood sugar level (Hr after injection of alloxan)										Degenerative changes in islets	
			Fasting	1	2	3	4	6	8	10	24	48	72	Ligated part	Nonligated part
519	16	60	72	108	74	92	120	44	91	—	152	197	215	+	+
419	22	200	100	84	98	107	113	128	116	116	61	456	912	+	+
524	20	75	94	110	106	132	110	90	64	78	102	240	276	+	+

None of these animals showed symptoms of hypoglycemic shock, none required glucose infusions and all survived and became diabetic. Similar experiments have been reported by Banerjee.[5] These observations seem to warrant the conclusion that the secondary alloxan hypoglycemia depends on the amount of islet cell tissue present or of insulin available. It seems to us that supportive evidence for this conclusion can be drawn also from the fact that the rabbit with its severe alloxan hypoglycemia has a relative high pancreatic insulin content (5-9 units per g) whereas in the dog the pancreas contains relatively less insulin (2-4 units per g) and the alloxan hypoglycemia is mild. We are, however, aware of the fact that this relationship between alloxan hypoglycemia and pancreatic insulin content does not hold true for every species and that the absolute weight of the pancreas and the rate of production of insulin may play a significant role.

Table IV shows results of experiments in which alloxan was given to dogs with partial ligation of the pancreatic duct. Walpole and Innes[6] in England have reported that duct ligation protects the islet cells against alloxan degeneration and have discussed the possibilities that either a normal acinar tissue may be necessary for the action of alloxan, or that the fibrosis prevents alloxan from reaching the islets—like clamping of the blood supply does. Our experiments rule out this latter mechanical possibility. We ligated only a part of the pancreatic duct, then permitted the ligated part to undergo fibrosis and after a period of 15-21 days gave a diabetogenic dose of alloxan. All 3 animals developed diabetes. Biopsies were taken from both parts of the pancreas and showed that the islet cell necrosis was equally severe in the fibrotic and in the normal part of the gland.

We have not performed total duct ligations. Pancreatic fibrosis, however, is not uncommon among dogs. Thus by coincidence we found a fibrotic pancreas in a dog which had received alloxan and 2 weeks later was pancreatectomized. This dog had developed alloxan diabetes as any other dog and histological examination made it very likely that the fibrosis had existed long before alloxan had been given.

Summary. (1) Further evidence has been presented to support the hypothesis that the secondary alloxan hypoglycemia is pancreatic in origin. (2) Fibrosis of part of the pancreas does not protect the islets in this part against the degenerative action of alloxan.

[5] Banerjee,, S., *J. Biol. Chem.*, 1945, **158**, 547.

[6] Walpole, A. L., and Innes, J. R. M., *Brit. J. Pharm. and Chemotherapy*, 1946, **1**, 174.

15851

Response of Experimental Hypertension to a Rice and Fruit Juice Diet.

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Dietary treatment of hypertension has received the attention of many investigators. Fishberg[1] in summarizing the evidence concluded that "No dietary treatment is known which has a specifically favorable effect on essential hypertension." More recently Kempner[2] has reported striking therapeutic results in a majority of patients with both "primary" and "secondary" hypertension by the use of a diet of rice, fruit, and fruit juices. Grellman, Harrison, and co-workers[3] have suggested that rigid sodium restriction is responsible for the changes observed by Kempner.

It is the purpose of this communication to record preliminary studies on 12 hypertensive dogs kept for 8 weeks on the Kempner regime. The hypertension in these animals had been produced by a nephrosclerosis which followed the intravenous administration of streptococci, as already reported by Dick.[4] At the time the present studies began, hypertension had been maintained for 2 to 4 years. Blood pressure was determined monthly throughout this period by direct arterial puncture with kymographic recordings through a mercury manometer.

Prior to the institution of the "rice" diet the animals had been fed on meat and dog biscuits provided approximately 900 calories per day. The basic experimental diet consisted of 170 g of rice, 340 cc of fruit juice, and 60 g of sugar. It contained approximately 13 g of protein and 212 g of carbohydrate with a total value of 900 calories. Daily supplements of 6000 I.U. of vitamin A, 1600 I.U. of vitamin D, 25 mg of nicotinamide, 1.6 mg of thiamine chloride, and .45 g of ferrous sulfate were administered. Since the animals often refused part of the diet offered, the basic diet represents the maximum possible intake. One dog refused the diet entirely and died before any significant observations were made.

TABLE I.
Blood Pressure Before and After Kempner Diets.

Dog No.	Mean arterial pressure, mm Hg.				Weight		
	Before induction of hypertension	Before beginning diet	After 8 wk of diet	Change	Initial, kg	Final, kg	% change
1.	105	220	138	—82	12.8	10.4	—18.7
2.	120	200	132	—88	16.4	14.2	—13.4
3.	132	210	154	—56	12.6	9.4	—22.2
4.	120	192	152	—40	12.4	8.6	—30.6
5.	120	168	126	—42	13.5	9.8	—27.4
6.	120	165	142	—23	10.2	9.3	— 8.1
7.	130	162	144	—18	12.4	8.6	—30.6
8.	120	160	124	—36	10.3	8.2	—18.4
9.	130	160	120	—40	8.2	5.8	—29.2
10.	120	150	148	— 2	10.2	9.3	— 8.9
11.	110	140	120	—20	9.4	9.7	+ 3.1
Avg	120.3	181.6	138	—43.6	12.1	9.7	—19.7

[1] Fishberg, A. M., *Hypertension and Nephritis*, p. 704, Lea and Febiger, Philadelphia, 1939.

[2] Kempner, W., *North Carolina M. J.*, 1945, **6**, 61; *ibid.*, 1945, **6**, 117.

[3] Grollman, A., Harrison, T. R., Mason, M. F., Baxter, J., Crampton, J., and Reichmann, F., *J. A. M. A.*, 1945, **129**, 533.

[4] Dick, G. F., *Arch. Path.*, 1945, **39**, 81.

As noted in Table I a significant reduction in arterial pressure occurred in 10 of the 11 dogs. The greatest fall in pressure was found in the animals with the highest initial levels. Although weight loss was evident, no direct quantitative correlation could be established between the amount of weight loss and the extent of fall in blood pressure.

The average blood nonprotein nitrogen was 23.6 mg per 100 cc before the diet and after 8 weeks had decreased to 18 mg per 100 cc. The average total plasma protein was 6.20 g per 100 cc initially and 6.22 g per 100 cc after 8 weeks.

It appears that the Kempner regime is capable of causing significant lowering of the arterial blood pressure of dogs made hypertensive through the induction of nephrosclerosis. The role of weight loss, salt restriction, and nitrogen balance in this result requires further study.

Summary. In 11 dogs with experimental hypertension the blood pressure fell in every animal following Kempner diets.

15852

Hypoglycemic Effect of Intraspinal Glucose Injection.

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We wish to report our experimental observations on variations in the glucose content of the blood produced by the introduction of glucose into the spinal fluid.

In diabetes the level of glucose in the spinal fluid is subject to considerable variation. Moreover some regions in the brain can be supplied with nutritive substances only through the cerebrospinal fluid. Not only may nutritional influences be exerted but stimulation of the central nervous mechanism may also take place, through variation of the chemical composition of the fluid.

The first series of experiments were performed on dogs. Blood and spinal fluid glucose levels were determined on the fasting animal and 0.1 g glucose was then injected directly into the "cisterna magna" after an equivalent amount of spinal fluid had been removed. The concentration of the injected glucose solution was calculated to increase the glucose content of the fluid to approximately twice the initial values. Blood and spinal fluid sugar levels were determined at frequent intervals by the Hagedorn-Jensen[1] method, since this requires only 0.1 ml of material. In previous control experiments it was established that (1) the blood glucose content of normal dogs showed practically no variation (± 3 —8 mg %); (2) neither the suboccipital tap alone, nor the introduction of isotonic salt solution, influenced the level of the blood sugar; (3) introduction into the spinal space of redistilled water had no effect upon the blood glucose level. This last control test was made in order to eliminate the possibility that the introduction of the glucose solution would produce an effect upon the nervous center by changing the ionic equilibrium.

After the introduction of the glucose solution, there was a steep rise in the level of the spinal fluid glucose followed by a rapid fall to the base level. The blood sugar reached its lowest level in 15 minutes and returned to normal after 2 hours (Table I).

The experiments were repeated in the same way in man. After fasting for 12 hours, 0.2 g of glucose was introduced into the spinal cavity to approximately double the initial level of glucose. Controls similar to those on the dog were repeated on man with the same negative results. Table II shows the results of these experiments.

[1] Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **137**, 92.

TABLE I.
Blood Sugar Levels Following Intraspinal Injections of Glucose in Dogs.

Dog No.	Blood sugar mg % after							
	0′	5′	10′	15′	30′	60′	90′	120′
1.	89	77	41	34	37	42	78	80
2.	92	86	75	35	66	74	83	90
3.	87	84	46	31	35	71	79	80
4.	91	72	50	29	38	74	83	95
5.	96	81	69	38	33	65	76	93

TABLE II.
Blood Sugar Levels Following Intraspinal Injections of Glucose in Normal Human Subjects.

Case	Blood sugar mg % after							
	0′	5′	10′	15′	30′	60′	90′	120′
P.D.	94	90	76	54	62	68	79	92
P.G.	101	92	79	57	65	73	96	97
T.R.	89	81	71	47	64	72	90	89
C.B.	99	90	88	62	64	77	95	94
O.D.	92	83	75	51	70	68	75	95

The increased level of the spinal fluid glucose depressed the blood sugar values to a minimum of 47-62 mg % within 15 minutes. These values returned to normal in 90-120 minutes. The findings in dog and man, therefore, showed an absolute parallelism.

The curves obtained look very much like those following insulin administration, with a steep fall in the first 15 minutes (the assimilation phase) followed by a gradual rise to normal (restoration phase). Thus we may infer that the increased glucose content of the cerebrospinal fluid represents a direct chemical stimulation upon the glyco-regulatory nervous centers, and that this is, in turn, followed by a series of functional changes affecting the glucose level in blood.

Summary. The experimental increase of spinal fluid glucose caused rapid changes in blood sugar values, with a severe hypoglycemia.

15853

Phosphorus Metabolism in Active and Inactive Nerves.

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Hecker[1] showed that *in vitro* the release of P of an isolated nerve is significantly increased when the nerve is stimulated electrically, and lately Cicardo[2] has found the P-content of the cephalic venous blood increased after a period of artificial hyperactivity of the brain *in vivo*. There is some evidence however that this P-release after artificial stimulation might have nothing to do with the normal activity of the nervous matter itself, but rather with an altered metabolism as a consequence of the "unphysiological" excitatory process. This is suggested by the fact that in the metabolism as a whole a remarkable difference between

[1] Hecker, E., *Hoppe Seyler's Z.*, 1923, **129**, 220.
[2] Cicardo, V. H., *Am. J. Physiol.*, 1946, **145**, 542.

artificially-induced activity and natural activity has been found (Winterstein).[3] For instance, Winterstein,[3] Lebedur[4] *et al.* found that the CO_2-production of nerves during electrical stimulation was higher than that of unstimulated ones, whereas no certain difference was found when the CO_2-production of normally active nerves was compared with that of nerves made inactive by cutting both ends (Parker).[5] On the other hand, however, Gerard and Hartline[6] were able to show that under certain experimental conditions the normal activity of a nerve (optic nerve of limulus) may produce about the same change in the oxygen consumption as the activity induced by an electrical stimulus. This change consists of an increase of about 40%.

In order to obtain further information about this problem the following experiments were performed:

Rabbits of about 2 kg weight were used under light Nembutal-anesthesia (the pupils were wide open, and reacted normally to light). One eye was then covered completely with a light-tight material, the other eye was kept exposed to normal daylight and artificial electrical light. Everything which might have irritated either of the eyes was carefully avoided. This experimental procedure provided a purely physiological activity or inactivity, respectively, and since the optic nerves practically can be considered as consisting of only visual fibers, normally active and inactive nerves under exactly comparable (same individual) and physiological conditions were obtained. Then the animals received a subcutaneous injection of radioactive phosphorus (P^{32}), the amount of which is indicated in Table I. After that they were kept under the above mentioned conditions for 5, 10 or 20 hours. They were then injected with heparin, killed by suffocation and immediately perfused through the aorta with 500-700 ml of a solution containing 0.85%

TABLE I.

Date	Covered eye	P^{32} injected per mg body wt	Killed after	(a) Absolute P^{32} content per mg tissue				Intravasc. fluid	(b) Relative P^{32} content (in %)*			
				Optic nerve		Optic tract			Optic nerve		Optic tract	
				left	right	left	right		act.	inact.	act.	inact.
8/13	r.	630 x 10-15 mg	5 hr.	90 x 10-15 mg	87 x 10-15 mg	47 x 10-15 mg	53 x 10-15 mg		100	97	100	89
13	r.	630 x "	"	143 x "	147 x "	73 x "	66 x "	33 x 10-15 mg	100	103	100	111
9/18	l.	530 x "	"	106 x "	106 x "	56 x "	63 x "	16 x "	100	100	100	113
8/19	l.	130 x "	10 hr.	23 x "	20 x "	13 x "	13 x "	0	100	115	100	100
19	l.	130 x "	"	13 x "	13 x "	7 x "	7 x "	0	100	100	100	100
9/11	r.	670 x "	"	186 x "	190 x "	93 x "	93 x "	20 x "	100	102	100	100
7/29	r.	370 x "	20 hr.	216 x "	210 x "	97 x "	97 x "	13 x "	100	97	100	100
29	l.	400 x "	"	160 x "	166 x "	90 x "	97 x "	10 x "	100	96	100	108
8/19	r.	420 x "	"	66 x "	63 x "	23 x "	26 x "	3 x "	100	95	100	88
								Avg	100%	101%	100%	101%

* The nervous matter belonging to the normal (*i.e.*, active) visual system is arbitrarily given the value 100%. Since the rabbits have virtually only crossed fibers, we have to consider that the right optic nerve belongs to the left optic tract and vice versa.

[3] Winterstein, H., *Pflueger's Arch.*, 1930, **224**, 749.

[4] Lebedur, J., *Pflueger's Arch.*, 1931, **227**, 343.

[5] Parker, C. H., *J. Gen. Physiol.*, 1928, **12**, 419.

[6] Gerard, R. W., and Hartline, H. K., *J. C. C. P.*, 1934, **4**, 141.

NaCl and 0.05% Na_2HPO_4, followed by 100-200 ml of 4% formaldehyde. Then both optic nerves (the anatomically well defined part from the bulb to the chiasma) and both optic tracts (anatomically as far as possible corresponding parts) were removed and weighed immediately. Then each of these 4 samples were pressed separately between 2 tin foils, the resulting layer being about 2 mg of nervous tissue per sq. cm. The tin foils, after being covered with a cellophane sheet of about 0.02 mm in thickness, were wrapped directly around the counter tube. In this way the rate of radioactivity was determined, and from the number of disintegrations per second the content of P^{32} per mg fresh nervous matter was calculated. In order to obtain a test of the sensitivity of the whole experimental procedure, we determined on the same rabbits the P^{32} content of the right and left dorsal roots S_2, taking anatomically corresponding pieces (from the ganglion to the cord) of about the same weight as the optic nerves. Working on the assumption that the P^{32} uptake herein is the same on both sides, we found a mean error of $\pm 4\%$ of the methodical procedure as a whole.

The data are given in Table I. The relatively great differences in the absolute values of certain rabbits, which are otherwise under the same conditions, are sufficiently explained by the fact that the perfusing process apparently was not equally efficient in every case. This becomes clear by a comparison of the values of the intravascular fluid which remained in the circulatory system after the perfusing process.

Table I shows that the values for the total phosphorus metabolism of the optic nerves (and optic tracts) are practically the same whether the optic system is in a stage of activity or not. This seems to be true for the average of all of the experiments as well as for each of the 3 groups calculated separately. And since the bulk of all P-compounds of a nerve belong to the nerve sheet of its fibers, our data may support the conception, that the metabolism of the nerve sheet is not immediately altered by the activity of the axis cylinder. Furthermore Table I shows that the phosphorus metabolism of the optic nerves is about twice as high as that of the immediately adjacent optic tracts. This is in accord with the fact that there are also remarkable histological differences (Schindler),[7] and that the total metabolism (measured by the CO_2-production) of a nerve becomes greater approaching to the nerve cell body (Tashiro).[8]

Conclusion. We may therefore conclude that there is no difference between the phosphorus metabolism of these active and inactive nerves.

Summary. No difference was found by means of P^{32} in the phosphorus metabolism of stimulated and unstimulated nerves.

7 Schindler, E., *Z. f. Augenheilk.*, 1926, 15.

8 Tashiro, S., and Adams, H. S., *J. Biol. Chem.*, 1914, **18**, 329.

15854

Studies on the Culture of *Endamoeba histolytica.**

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Endamoeba histolytica has been grown in culture for many years.[1] Little is known, however, concerning the metabolic requirements of this organism or of the true functions of bacteria which so far appear to be necessary to the *in vitro* cultivation of this

* Supported by a grant from the Parke-Davis Company, Detroit.

1 Boeck, W. C., and Drbohlav, J., *Am. J. Hyg.*, 1925, **5**, 371.

protozoan. Previously-used culture media are unusually complex. In order that the metabolic requirements of *E. histolytica* and the relationships of its associated bacteria could be more fully studied, our efforts have been directed to the development of a simple medium. This paper reports such a medium and the presence of a growth factor in human serum which is essential to the propagation of *E. histolytica* under the cultural conditions described herein.

Conditions and Method. The cultivation of *E. histolytica* entails consideration of numerous variables; the strain of ameba, the associated flora and the basic conditions of culture. These factors have been controlled in so far as possible.

1. Strain of *E. histolytica.* The organism used in these experiments was the NRS strain, originally obtained for use by this department from Dr. Henry E. Meleney. Used for comparative studies, was a strain obtained from Dr. Charles W. Rees of the National Institute of Health.

2. Bacterial flora. The bacterial flora present in our culture of the NRS strain includes a diphtheroid, *Staphylococcus aureus, Escherichia coli,* a gamma streptococcus, and a Gram-variable, branching bacillus which has not yet been identified. The only bacterium present in Rees' strain is the so-called "organism T."[2]

3. Culture media. Raw egg white was selected as a starting point following numerous attempts to grow amebae on various parts of fertile eggs. The constituents of egg white are largely known,[3] and it was believed that this substance, therefore, would constitute a suitable base for a medium. The basic medium used in this work is a buffered infusion of egg white to which has been added rice starch and either whole human serum or its protein-free filtrate.

(a) Egg white infusion medium: To egg white, collected in a sterile manner, is added sterile M/15 phosphate buffer, pH 6.8, in a ratio of 3 parts of buffer to one part of egg white. The mixture is allowed to infuse, with occasional shaking, at 4°C for at least 24 hours before decanting the supernate from any thick albuminous sediment which fails to dissolve.

(b) Rice starch. The rice starch used was obtained from Dr. Kessel's laboratory. It consists of husk-free starch granules approximately 1.5 μ in size. After sterilization by dry heat at 180°C, for one hour, it is added by loopfuls, as necessary, to each culture tube.

(c) Human serum. Pooled Wassermann-negative serum, obtained from the Serology Laboratory at the Los Angeles County Hospital, has been used in these experiments. It is Seitz-filtered and kept in sterile containers at 4°C until used.

(d) Protein-free fraction of human serum. At first, partially deproteinized serum was prepared by heating serum at 100°C for 20 minutes in a water bath. More recently, all protein has been removed by acidifying the serum to pH 5.2 and heating, as before. This procedure appears to remove all precipitable protein.

(e) Dialysate. The protein-free fraction of human serum was dialyzed with 2 volumes of 0.9% saline solution for a 24-hour period.

4. Assessment of growth. Accurate assessment of multiplication of amebae in culture is frequently very difficult. In testing whether or not a constituent of the medium was vital to propagation, results have been interpreted on a basis of presence or absence of amebae. This, in turn, required differentiation between "survival of the inoculum" and "multiplication." If amebae persist in cultures in fairly constant numbers through serial dilution of numerous subcultures, they must propagate. When they survive without propagation in serial dilution through subculture, we have observed that cultures become negative between the 2nd and 6th transplants. Continued presence of amebae beyond the 6th transplant has therefore been considered significant.

Description of Results. 1. Egg white infusion medium containing rice starch with

[2] Rees, C. W., and Reardon, L. V., *J. Parasit.*, Suppl., 1944, **30**, 10.

[3] McNally, E. H., and Denton, C. A., *Composition of Hen's Eggs,* A compilation Bureau of Animal Industry, U. S. D. A.

human serum added in dilution of 1:10 has supported growth of the NRS strain of *E. histolytica* since December 1946. Transplants have been made at 48-hour intervals.

2. Egg white infusion medium containing rice starch with partially deproteinized human serum added in a dilution of 1:10, has thus far supported growth of the NRS strain of *E. histolytica* through 42 transplants for 66 days.

3. Egg white infusion medium containing rice starch with completely deproteinized human serum added in a dilution of 1:10 has thus far supported growth of the NRS strain of *E. histolytica* for 38 days through 27 subcultures.

4. Egg white infusion medium containing rice starch with dialysate of the protein-free fraction of human serum added in a dilution of 1:5 has so far supported growth through 7 transplants for 10 days.

5. Egg white infusion medium containing serum or its protein-free fraction but *without rice starch* has failed to support growth of *E. histolytica* beyond the 3rd subculture. We have observed that there is a more rapid rise in the bacterial population in the absence of starch than when starch is present.[4] The cause for this may only be implied. Rice starch appears to suppress bacterial proliferation at least temporarily. In our hands, excessive growth of bacteria is detrimental to amebae in culture and amebae die out. If, however, bacterial multiplication is restricted by starch, as in these experiments, more viable amebae are found. This mechanism is being studied further.

Rice starch is likewise actively phagocytosed by *E. histolytica* and, as Boeck and Drhbolav[1] described, its presence enhances proliferation of amebae in culture.

6. Egg white infusion medium containing rice starch but without human serum or its protein-free fraction, uniformly fails to support growth of this strain of *E. histolytica*. However, growth of the accompanying bacterial flora is uninhibited.

These experiments demonstrate the presence of a growth-promoting substance in human serum necessary to the propagation of the NRS strain of *E. histolytica*, under these cultural conditions. This substance resists heating at 100°C for 4 hours and is dialyzable.

7. The partially deproteinized fraction of human serum diluted with an equal quantity of M/15 phosphate buffer with rice starch added constitutes, in itself, an excellent medium for the cultivation of *E. histolytica*. Growth has so far been supported through 35 subcultures for 50 days. Various dilutions of the protein-free fraction have also been tried. A 1:10 dilution also supports growth well. Analysis of the constituents remaining in this fraction is being made.

8. Growth of Rees' strain of *E. histolytica*, with organism "T," has likewise been tried in the protein-free human serum fraction diluted in an equal volume of M/15 phosphate buffer containing rice starch. No amebae survived beyond the third day. However, when the bacteria, accompanying our NRS strain, were added to Rees' strain, rapid proliferation occurred which has now been maintained for 23 days through 15 subcultures. It is interesting that under identical cultural conditions, Rees' strain and the NRS strain of *E. histolytica* behave in a similar manner in the presence of identical enzyme systems contributed by the bacteria present. This suggests that cell variation and culturability of *E. histolytica* may depend largely upon the bacteria present at the time of isolation. Detailed study of the biology of the accompanying bacteria may furnish more exact information concerning the physiologic requirements of *E. histolytica, in vivo* and *in vitro*.

Summary and Conclusions. 1. A heat-stable (100°C for 4 hours), dialyzable substance has been demonstrated in the protein-free fraction of human serum. This substance is essential to the growth of the NRS strain of *E. histolytica* in egg white buffer infusion medium containing starch. 2. Par-

[4] Balamuth, W., and Howard, B., *Am. J. Trop. Med.*, 1946, **26**, 771.

tially deproteinized filtrate of human serum diluted in an equal volume of M/15 buffer, with rice starch added, constitutes a simple medium for the propagation of *E. histolytica*. 3. Rice starch appears to act as a temporary inhibitor to bacterial multiplication in these cultures. Such suppression leads to more satisfactory growth of the amebae present. 4. Study of the biology of the bacteria in these cultures may give further information concerning the complex metabolic requirements of *E. histolytica*.

15855

Redistribution of Residual Blood Volume in Hemorrhagic Shock; Relation to Lethal Bleeding Volume.*

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When unrestricted hemorrhage is permitted from a major artery bleeding proceeds until death eventuates; the total amount of blood that has flowed out is called the *bleeding volume*. When death occurs hemorrhage has either ceased or is reduced to a negligible ooze even though a considerable amount of blood obviously still remains in the body; this remainder is called the *residual volume*. It is known that bleeding volume of animals is less than normal in shock, an observation that has often been used as evidence that the total blood volume is reduced in shock. The object of this study was to determine the magnitude of the residual volume and its distribution in the body after hemorrhagic shock as compared to that of normal animals.

Methods. Dogs weighing 7.9 to 23.3 kg were anesthetized with morphine sulfate and sodium barbital. Both femoral arteries and a femoral vein were cannulated for the purposes of bleeding, reinfusion, and the continuous recording of mean arterial blood pressure with a mercury manometer. A tracheal cannula was inserted. For determining the bleeding volume in the control animals blood was allowed to flow freely from a femoral artery cannula at the rate of 2 cc/kg/minute until bleeding ceased and/or death ensued. These events were virtually simultaneous in all cases. For producing hemorrhagic shock the animals were bled so that mean arterial pressure was maintained at 50 mm Hg for 90 minutes and then at 30 mm Hg for 45 minutes more, after which all withdrawn blood (heparinized, warmed, and filtered) was reinfused. The bleeding volume of the shock animals was determined in this way: One hour after reinfusion, hemorrhage was begun at the rate of 2 cc/kg/minute and was continued in the same manner until death as for the control animals.

After death the residual volume was determined without delay. The femoral cannulae were tied off. The chest was opened by midline thoracotomy. With suitable precautions to prevent blood loss the inferior vena cava was sectioned about 1 cm distal to the right atrium. Two cannulae were inserted; one distally into the inferior vena cava and the other proximally toward the atrium and the superior vena cava. The atrium was clamped across so as to occlude the right atrio-ventricular orifices. Through this arrangement as much venous blood as possible was collected by the upper cannula from the region of the body drained by the superior vena cava and the azygos vein, while blood from regions drained by the inferior vena cava was collected from the lower cannula. The blood still remaining in these 2 territories was next washed out. A cannula inserted through the wall of the left

* Supported by a grant from the Commonwealth Fund.

† Research Scholar in Physiology.

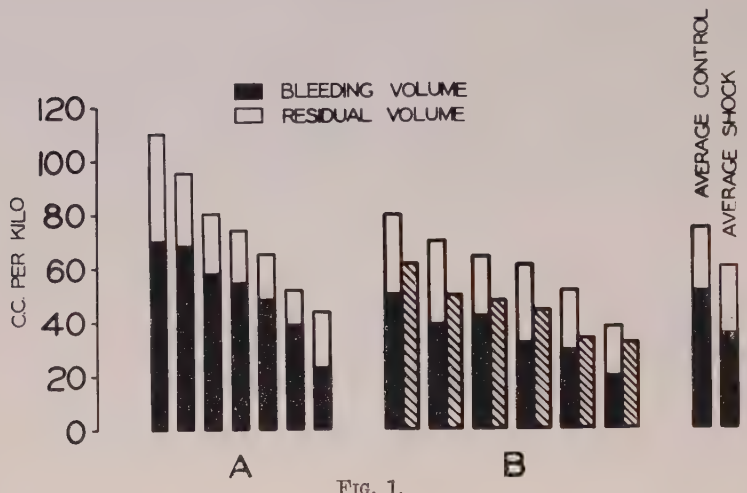

FIG. 1.

ventricle was passed through the aortic valve into the aorta; this was held in place by a ligature around the aorta. A solution of 0.9% NaCl and 1.0% sodium citrate was introduced into the aortic cannula under a constant pressure of 150 mm Hg. The mixture of blood and saline flowing from the 2 cannulae was collected separately. Perfusion was continued until the perfusate was clear and free of blood grossly. The oral and intestinal mucosa and other tissues examined after perfusion were invariably white; various muscles and the liver and spleen were relatively pale; no blood or bloody fluid could be expressed. In a few cases veins of the mesentery contained a light pink fluid indicating the admixture of a small quantity of blood; this was not regarded as significant. The blood still remaining in the right ventricle, pulmonary vessels and left atrium was drained as follows: A cannula was inserted into the left auricular appendage for drainage. The superior vena cava was clamped and the A-V clamp removed. By connecting the cannula in the central end of the inferior vena cava to a perfusion bottle blood was washed out.

The amount of blood contained in the perfusates was estimated through use of the conventional acid hematin method. A sample of arterial blood was taken from the animal just before death; it is assumed that the hemoglobin content of this sample was the same as that of the residual blood. The blood sample was diluted with the saline-citrate perfusion fluid so as to give a known dilution of the same magnitude as that estimated for the perfusate. Acid hematin was formed by the addition of hydrochloric acid in amount so as to give a final concentration of 0.1 normal. (It was determined that the saline-citrate solution did not affect the formation of acid hematin). Four or more different dilutions of this known solution were tested for light absorption in a Coleman spectrophotometer using a 450 γ filter. The curve plotted for these values became the reference standard against which the perfusate was compared. Appropriate amounts of the perfusate were prepared as above and absorption was determined. The concentration of hemoglobin in the perfusate was then calculated as compared to a concentration of 1.0 in the whole blood sample. This factor multiplied by the perfusate volume equals the volume of blood contained in the perfusate.

Results. Fig. 1-A shows the results obtained from 7 control animals, and Fig. 1-B those from 6 shock animals. (Eight experiments performed during development of technics are not reported here; their results, however, were consistent with those reported).

As shown in Fig. 1-A, bleeding volume in the control animals varied from 22.8 cc/kg to 70.7 cc/kg with an average of 51.4 cc/kg; residual volume varied from 12.9 cc/kg to 39.0 cc/kg with an average of 22.5 cc/kg;

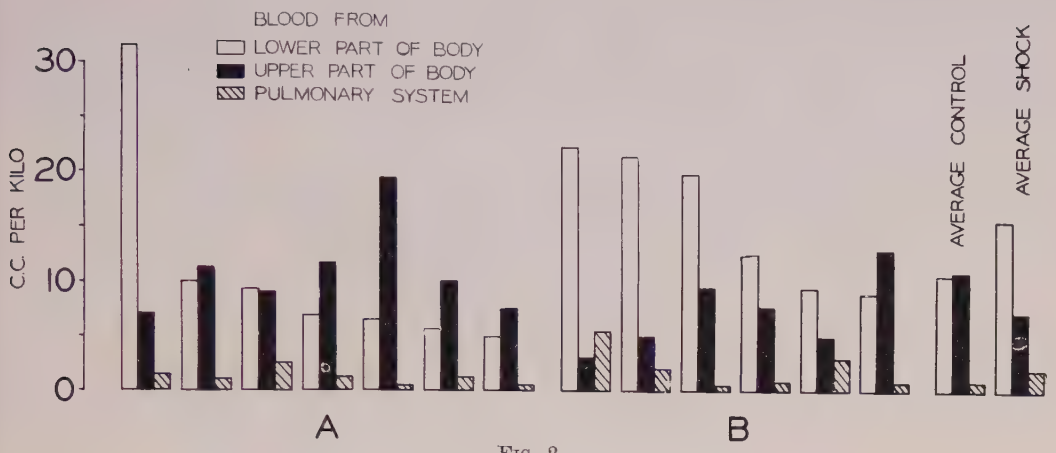

FIG. 2.

and the total blood volume varied from 43.5 cc/kg to 109.7 cc/kg with an average of 73.9 cc/kg. Fig. 1-B shows that the bleeding volume for the shock animals varied from 19.5 cc/kg to 50.2 cc/kg with an average of 35.2 cc/kg; residual volume varied from 17.9 cc/kg to 31.0 cc/kg with an average of 25.2 cc/kg; and the total blood volume varied from 37.4 cc/kg to 80.0 cc/kg with an average of 60.5 cc/kg. Results from the control animals are compared with those for the shock animals in tabular form below.

	Control	Shock
Avg total volume	73.9 cc/kg	60.5 cc/kg
Avg bleeding volume	51.4 "	35.2 "
% of total	69.2%	57.6%
Avg residual volume	22.5 cc/kg	25.2 cc/kg
% of total	30.7%	42.3%

The total blood volumes observed presumably represent the actual circulating volumes since perfusion should be unaffected by cardiodynamic alterations. While they are, on the average, less in shock dogs, this does not permit the conclusion that the total blood volume in any particular animal is reduced as a result of hemorrhagic shock. It is well established that the blood volumes per kg body weight of different dogs varies considerably. Moreover, Overbey *et al.*[1] recently reported that blood volumes estimated by dye methods decreased by only 0.7 ± 1.64 cc/kg during development of a similar type of hemorrhagic shock.

1 Overbey, D. T., Ramirez, A., Wiggers, C. J., and Lawson, H. C., *Fed. Proc.*, in press.

The difference between the average bleeding volumes is considered significant. The bleeding volume is lower in the shock animal or, stated in another way, the heart pumps out a smaller percentage of available blood before the animal dies. There is, of course, a corresponding difference between the average residual volumes. Assuming that the total blood volumes are of the same order these data show the expected; namely, that blood which the heart of the shock animal cannot pump out of the body simply remains within it.

Fig. 1-B also shows the amount of blood withdrawn in order to lower the mean arterial blood pressure to 30 mm Hg during the initial hemorrhage (cross hatched bars). This volume varied from 31.6 cc/kg to 61.9 cc/kg with an average of 44.7 cc/kg. It is obvious that in every experiment this value exceeded the lethal bleeding volume after shock had developed.

The distribution of the residual volume is shown in Fig. 2-A for the control animals and in Fig. 2-B for the shock animals. In these graphs the volumes obtained respectively from the superior vena cava-azygos system, from the inferior vena cava system, and the cardiopulmonary system are compared at a glance.

It is obvious that as a rule the residual superior cava-azygos volumes exceed the inferior caval volumes in control animals. On the contrary, in every experiment except one

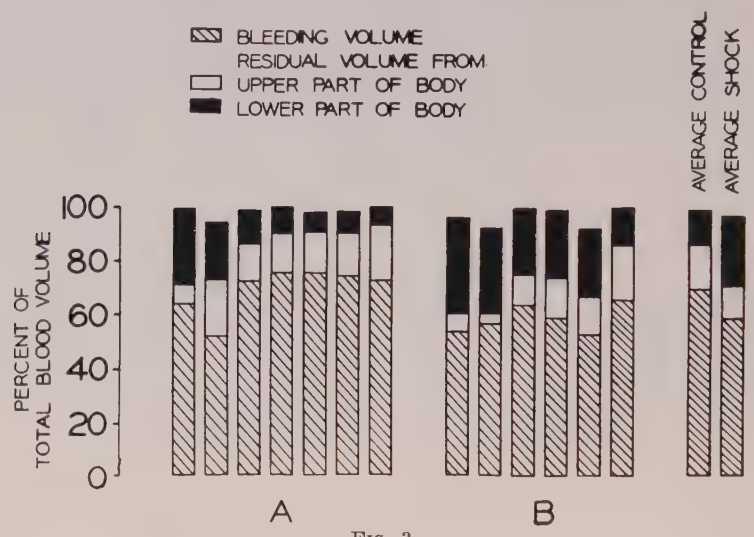

FIG. 3.

the reverse relation existed after development of shock. The pulmonary blood volumes were of such small magnitude that evaluation of differences becomes hazardous. While a little more blood seems to stagnate in the cardio-pulmonary circuit this is very insignificant compared to the great increase in the inferior caval territory. Results are compared in tabular form below.

	Control	Shock
Avg residual volume	22.5 cc/kg	25.2 cc/kg
Avg SVC-azygos volume	10.8 "	7.2 "
% of residual volume	48.0%	28.5%
Avg IVC volume	10.6 cc/kg	15.8 cc/kg
% of residual volume	47.1%	62.7%

(Note: Residual blood not accounted for above was recovered from the pulmonary system.)

Finally, all of the data obtained in this study are integrated in Fig. 3 as percentages of the total blood volume. They show that the residual volume remaining in the lower part of the body has increased proportionately, partly at the expense of the residual volume from the upper part of the body, but chiefly at the expense of the bleeding volume. It is obvious that bleeding volume is not a reliable criterion of total blood volume after development of shock.

Discussion. Reviews of recent literature[2,3] indicate that while reduction in blood volume is a concomitant of most common forms of shock, this is not the sole factor in development of irreversible circulatory failure. Furthermore, the development of shock without significant reduction in blood volume is not precluded. According to studies of Overbey *et al.*,[1] this is the case in a type of hemorrhagic shock which follows reinfusion of all blood withdrawn during preceding hemorrhage (standardized hemorrhagic shock procedure used in this laboratory). In this type of shock blood tends to pool in splanchnic areas as indicated by direct observations of intestinal capillaries,[4] development of passive congestion, edema, and hemorrhage in the intestinal mucosa,[5] persistence of high portal pressure,[6] and an apparent reduction in resistance in the mesenteric circuits.[7] With such premortal pooling in the splanchnic organs it may be expected that lethal bleeding volumes are reduced, not by virtue of a reduction in total blood volume but because it is not mobilized from regions of pooling before death from bleeding takes place. The partition of residual blood after lethal hemor-

2 Gregersen, Magnus, I., *Ann. Rev. Physiol.*, 1946, **8**, 335.

3 Wiggers, C. J., *Ann. Rev. Physiol.*, 1947, **9**, 278.

4 Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, 1944, **120**, 232.

5 Werle, J. M., Cosby, R. S., and Wiggers, C. J., *Am. J. Physiol.*, 1942, **136**, 401.

6 Wiggers, C. J., Opdyke, D. F., and Johnson, J. R., *Am. J. Physiol.*, 1946, **146**, 192.

7 Selkurt, E. E., *Am. J. Physiol.*, in press.

rhage in shock dogs reported in this investigation supports this interpretation.

Summary. 1. Seven control dogs anesthetized with morphine sulfate and sodium barbital were bled to death at the rate of 2 cc/kg/minute. Six dogs similarly anesthetized were first subjected to a standardized hemorrhagic shock procedure and were then reinfused with all blood previously withdrawn. They were bled to death 60 minutes after reinfusion at the same rate as the normal dogs. In each instance, the residual blood volume was determined after death by perfusion with a sodium chloride-sodium citrate solution under 150 mm Hg pressure. The residual volume was partitioned into fractions obtained from (1) the superior cava-azygos, (2) the inferior cava, and (3) the cardio-pulmonary systems.

2. The average total blood volume of the control animals slightly exceeded that of the shock animals, but the difference is not believed significant. The bleeding volume was significantly greater in the control animals, and correspondingly the residual volume was greater in the shock animal.

3. There was a significant shift in the distribution of the residual volume in the animal dying in shock. As compared to the controls, there was a marked increase in the residual volume of blood retained in the inferior vena cava territory and an accompanying decrease in the superior cava-azygos system.

4. The conclusion is reached that the reduced lethal bleeding volumes after transfusion of dogs in hemorrhagic shock are not significantly due to reduction in total blood volume but to pooling of greater volumes of residual blood in the splanchnic vessels. Changes in the cardio-pulmonary residual volumes are too small to affect lethal bleeding volumes.

The author wishes to thank Dr. C. J. Wiggers and Dr. D. F. Opdyke for their guidance and suggestions in this investigation, and to Mr. D. G. Pocock for his technical assistance.

15856

Anesthetic Action of Beta-Dimethylaminoethyl Benzhydryl Ether Hydrochloride (Benadryl) in the Skin of Human Beings.*

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Beta-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) is one of the recent members of a series of synthetic antihistamine compounds which currently are receiving clinical trial and are showing promise of usefulness in the treatment of some types of allergy.[1-7] In regard to anesthetic action, only the first of this series of compounds, thymoxyethyldiethylamine (929 F), has been studied in detail. This compound was shown by Bovet and Staub[8,9] to possess antihista-

* Abridgment of thesis submitted by Dr. Leavitt to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of M.S. in Medicine.

1 McElin, T. W., and Horton, B. T., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 417.

2 O'Leary, P. A., and Farber, E. M., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 429.

3 Koelsche, G. A., Prickman, L. E., and Carryer, H. M., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 432.

4 Williams, H. L., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 434.

5 Logan, G. B., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 436.

6 Code, C. F., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 439.

7 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

8 Bovet, D., and Staub, A.-M., *C. R. Soc. de biol.*, 1937, **124**, 547.

9 Staub, A.-M., and Bovet, D., *C. R. Soc. de biol.*, 1937, **125**, 818.

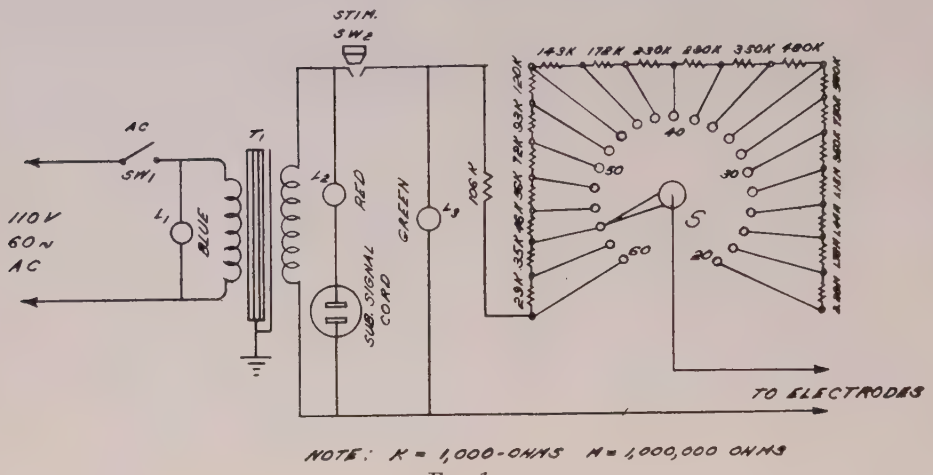

FIG. 1.

Schematic diagram of circuit of algesimeter. T_1, isolating transformer 1:1 ratio; S, calibrating attenuator for adjusting current; L_1, blue pilot lamp indicating current to the primary of T_1; L_2, red pilot lamp which lights when subject signal button is pushed; L_3, green pilot lamp which lights when stimulating switch SW_2 is closed.

mine and antianaphylactic properties. The anesthetic action of thymoxyethyldiethylamine was discussed by Staub[10] who noted also the marked intolerance of various animal species, including man, to its administration both locally and by mouth. Burchell and Varco[11] observed that large doses of the drug given subcutaneously to dogs produced a general analgesic effect which, according to their tests, was incomplete. In the skin of man they found no evidence of local anesthesia after subcutaneous injection, while intracutaneous injection produced anesthesia only in the raised portion of the epidermis. Rosenthal and Minard[12] noted a generalized elevation of the cutaneous threshold for pain in dogs which received thymoxyethyldiethylamine, and Rosenthal, Minard and Lambert[13] obtained similar results when studying visceral sensation in the dog. During these investigations it was reported that thymoxyethyldiethylamine in 0.5% solution, when given intracutaneously, possessed the anesthetic potency of a 1.0% solution of procaine.[12] Climenko, Homburger and Messer,[14] studying the pharmacologic action of thymoxyethyldiethylamine, noted that extremely inflammatory reactions, erythema, edema, necrosis and sloughing were produced in the skin when the drug was administered subcutaneously to mice, a result which confirmed similar studies made by Staub.

During an investigation of the antihistamine action of β-dimethylaminoethyl benzhydryl ether hydrochloride, hereafter referred to as benadryl, in the skin of human subjects, it was found that this drug also exhibits a pronounced local anesthetic action. A study then was undertaken to determine the effectiveness of the compound as a local anesthetic agent in the skin and to compare its potency in this regard with that of procaine.

Methods and Procedures. The anesthetic action of benadryl injected into the skin was tested in 10 healthy men and women whose ages ranged from 18 to 30 years. All tests were made at a constant room temperature

10 Staub, A.-M., *Ann. Inst. Pasteur*, 1939, **63**, 400, 485.

11 Burchell, H. B., and Varco, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 1.

12 Rosenthal, S. R., and Minard, David, *J. Exp. Med.*, 1939, **70**, 415.

13 Rosenthal, S. R., Minard, David, and Lambert, Edward, PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 317.

14 Climenko, D. R., Homburger, E., and Messer, F. H., *J. Lab. and Clin. Med.*, 1941, **27**, 289.

of 78°F and a relative humidity of 40%. The subjects lay supine on comfortable beds in the presence of minimal extraneous stimuli during the tests and for at least 30 minutes before the beginning of the tests.

Pain thresholds were determined by use of a simple type of algesimeter. The apparatus consisted of the necessary components to convert 110 volt, 60 cycle alternating current to an adjustable constant current at the subject electrodes. The current range of the device was from 10 microamperes to 1 milliampere subdivided in 20 steps such that each step gave approximately 26% (2 db) greater current than the preceding lower step (Fig. 1). Stimulation was applied by means of a smaller monopolar electrode approximately 3 mm in diameter; the large indifferent electrode was held in the hand of the subject on the side opposite to that being tested for anesthesia. The stimulating electrode was applied firmly but with minimal pressure to the skin.

An area approximately 2 by 12 cm midway between the elbow and the wrist on the flexor surface of either forearm was used in all tests. All stimuli were applied for one second and repeated 2 times with 3-second intervals between periods of stimulation. The subject then reported on the presence or absence of pain. As a rule, groups of 3 stimuli were repeated each minute, throughout the test. The strength of stimulus which was uniformly painful over the designated area was first established. Injections were then given and their effect on the sensation elicited by the threshold stimulus of the area determined. To assist the subject, sensation in intact skin was repeatedly compared with that in injected skin. When no sensation was felt the area was regarded as anesthetized. In most tests, as confirmation of the presence of anesthesia, one or two stimuli of 400 microamperes were applied. In many instances, as anesthesia subsided a period of altered sensation occurred during which stimuli sufficient to produce pain in normal skin were perceived as dull or blunt but not painful sensations in injected skin.

All injections were made intracutaneously, the amount of each was 0.3 cc and all were delivered from a 1 cc tuberculin syringe through a 27-gauge needle. Each injection produced a wheal about 1.5 to 2 cm in diameter. All dilutions of drugs to be injected into the skin were prepared in physiologic saline solution.

Since both benadryl and procaine were to be given intracutaneously, it was first necessary to determine the effect of the intracutaneous injection of an inert solution on cutaneous sensation. For this purpose physiologic saline solution was used and pain thresholds in normal skin and in skin injected with saline solution were determined on 3 successive days for each of the 10 subjects. Then the effects of benadryl and procaine were studied. In each test a similar procedure was followed. On one forearm a control wheal was made by injection of saline solution and 5 wheals were made by injection of benadryl in dilutions of 1:500, 1:1,000, 1:5,000, 1:10,000 and 1:20,000. Sensation in the benadryl-induced wheals was compared to that in the surrounding intact skin and in the control wheal induced by injection of physiologic saline solution. Each wheal was tested every 60 to 90 seconds. The duration of complete anesthesia and the duration of altered sensation were noted for each dilution of benadryl. When these tests were completed, a similar series was carried out on the other forearm, using procaine in dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600 and 1:3,200.

To determine the local irritant effects of benadryl, each of the 10 subjects received intracutaneous injections of 0.3 cc of benadryl in dilutions of 1:100, 1:500 and 1:1,000. Occurrence of immediate local reactions was noted and daily observations were made until all effects had subsided.

Results. Control determinations of pain thresholds. Control tests of pain thresholds were made on 3 successive days on the forearms of each subject. The minimal stimulus which consistently gave a sensation of pain in the forearms was quite constant from day to day in each subject, although there was considerable variation between subjects. In

TABLE I.
Comparison of Anesthetic Properties of Benadryl and Procaine.
(Both drugs and all dilutions tested on the same 10 subjects.)

	Mean duration of action in minutes					
	Procaine in dilutions of					
Action	1:100	1:200	1:400	1:800	1:1,600	1:3,200
Anesthesia	15.8 ± 0.7*	12.2 ± 0.6	9.8 ± 0.4	5.4 ± 0.6	0.3 ± 0.2	
Altered sensation	7.5 ± 1.5	6.4 ± 0.7	5.3 ± 0.7	5.0 ± 1.0	6.0 ± 0.7	2.1 ± 0.8
Total effect	23.3 ± 1.6	18.6 ± 0.8	15.1 ± 0.8	10.4 ± 0.8	6.3 ± 0.7	2.1 ± 0.8

	Benadryl in dilutions of				
Action	1:500	1:1,000	1:5,000	1:10,000	1:20,000
Anesthesia	12.3 ± 1.2	9.2 ± 0.6	2.8 ± 0.6	0.6 ± 0.2	
Altered sensation	6.4 ± 1.0	5.8 ± 0.6	7.4 ± 1.1	5.1 ± 1.1	2.4 ± 0.7
Total effect	18.7 ± 1.8	15.0 ± 0.9	10.2 ± 0.8	5.7 ± 1.0	2.4 ± 0.7

* The figure after the ± is the standard error of the mean.

the group of 10 subjects the strength of stimulus required consistently to produce pain ranged from 100 to 158 microamperes, with a mean value of 130.7 ± 8.1 microamperes for the group. The intracutaneous injection of physiologic saline solution did not appreciably alter the pain threshold of any of the subjects studied. An instrument capable of a finer adjustment than that of 26% in the strength of stimuli might have indicated some effect.

Anesthetic action of benadryl. In dilutions ranging up to 1:5,000, benadryl consistently produced complete anesthesia. On the average, the anesthetic effect of benadryl in dilutions of 1:500, 1:1,000 and 1:5,000 lasted about 12 minutes, 9 minutes and 3 minutes, respectively (Table I). Injection in dilution of 1:10,000, on the other hand, produced anesthesia in only half the subjects which lasted for only one or 2 minutes; all subjects, however, experienced some altered sensation which persisted for about 5 minutes. Given in dilution of 1:20,000 benadryl did not produce anesthesia in any of the subjects, although in the majority sensation was altered for some minutes.

Anesthetic action of procaine compared with that of benadryl. Procaine in dilutions up to 1:800 consistently caused complete anesthesia in the area in which it was injected (Table I). In general equianesthetic effects were produced by benadryl 1:500 and procaine 1:200; benadryl 1:1,000 and procaine 1:400; benadryl 1:5,000 and procaine 1:800; benadryl 1:10,000 and procaine 1:1,600, and, finally, benadryl 1:20,000 and procaine 1:3,200 (Fig. 2).

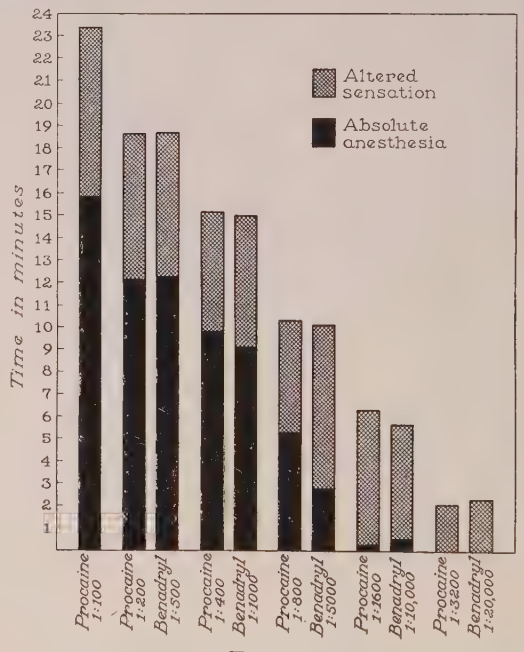

FIG. 2.
Comparison of local anesthetic effect of benadryl and procaine when injected into the skin of human subjects. Height of column gives average duration of anesthesia and altered sensation in the skin of 10 subjects after injection of the drugs in various dilutions.

Irritant effects of benadryl. Results of tests performed by injection of benadryl intracutaneously indicated that the drug possesses definite irritant properties when administered in concentrations greater than 1:500 (2 mg per cubic centimeter). Six of the 10 persons to whom benadryl in 1:100

dilution was given presented an immediate reaction at the site of injection; the reaction consisted of marked, burning pain which lasted several minutes and of diffuse local edema with pronounced redness. Four of these 6 individuals exhibited a severe delayed reaction in which sloughing and ulceration occurred at the site of injection. The ulcers, which never exceeded 1 cm in diameter, were superficial and painless; they healed within 10 days. In the other 2 of the 6 subjects the local reaction subsided within 24 hours without evidence of necrosis. Injection of benadryl in dilution of 1:100 caused no reaction in 4 subjects.

Administration of benadryl intracutaneously in dilution of 1:500 caused transient burning pain, redness and slight edema in 2 subjects; this reaction disappeared in 4 to 6 hours. Both of these subjects also had experienced a severe local reaction after administration of benadryl in dilution of 1:100. Eight subjects did not present signs of cutaneous irritation after injection of benadryl in dilution of 1:500.

None of the 10 subjects exhibited more than transient local redness in response to injection of benadryl in dilution of 1:1,000, although 3 reported the occurrence of mild burning pain, lasting only one or 2 minutes, at the time of injection.

Comment. As pointed out previously by others, methods for determining the local anesthetic effects of a drug are not entirely satisfactory. The results obtained may vary widely with the method used. Sinha,[15] after investigation of various technics used in the bio-assay of anesthetic agents designed for local administration, concluded that the human wheal method afforded greatest accuracy and was one of the most practical procedures for determination of anesthetic effect. Chance and Lobstein[16] have reported that quantitative inaccuracies of this method render it unsuitable for bio-assay of drugs, yet agree that the technic is of qualitative value in that it immediately discloses whether or not a given drug acts in the living body and also allows one to detect the occurrence of undesirable side effects. In the human wheal method the anesthetic agents act on the most minute nerve fibrils and nerve endings. Although the end-point in this method, the absence of sensation, is not too delicate and is entirely subjective in nature, it was sufficient for the purposes of this study of the relative anesthetic potency of benadryl and its irritant effects when introduced into human skin. Also, with a change of 26% in the strength of stimulus delivered by each adjustment of the algesimeter, the stimulus which consistently gave a pain response in the skin of the forearm remained quite constant from day to day and no differences between normal skin and skin infiltrated with saline solution were noted. An instrument which would permit finer adjustments of current strength might have indicated some variability.

It is possible that injections of benadryl in concentrations greater than 1:500 may produce anesthesia by virtue of injury to the tissue at the site of injection. However, in those wheals produced by intracutaneous injection of relatively dilute solutions of benadryl, the anesthetic reaction was reversible; complete return to normal sensitivity occurred at time intervals ranging from 2 to 18 minutes, depending on the concentration of drug employed. Local anesthetic action in the skin is, therefore, a definite property of the drug. Because of the irritant effects produced by benadryl administered locally it is unlikely, however, that this drug will have practical value as a local anesthetic agent.

In this study no correlation between anesthetic and antihistamine properties has been made. Do other antihistamine compounds possess anesthetic properties? Ray and Rieveschl[17] suggested the possibility that anesthetic properties might be possessed by a group of alkamine compounds which they had synthesized and the fundamental chemical structure of which is similar to that of

[15] Sinha, H. K., *J. Pharm. and Exp. Therap.*, 1936, **57**, 199.

[16] Chance, M. R. A., and Lobstein, H., *J. Pharm. and Exp. Therap.*, 1944, **82**, 203.

[17] Ray, F. E., and Rieveschl, George, Jr., *J. Am. Chem. Soc.*, 1943, **65**, 836.

benadryl. The possibility of correlation between anesthetic action and antihistamine effect deserves further study.

Summary. A study was made of the local anesthetic effect of β-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) when injected into the skin of human beings. The results were as follows: 1. By means of electric algesimetric determinations, benadryl in dilutions of 1:500, 1:1,000, 1:5,000, 1:10,000 and 1:20,000 was found to possess anesthetic potencies similar to those of procaine in dilutions of 1:200, 1:400, 1:800, 1:1,600 and 1:3,200 respectively. 2. Benadryl in concentrations greater than 1:500 (2 mg per cubic centimeter) proved to be exceedingly irritating when injected into human skin, causing tissue necrosis and ulceration in 4 of 10 subjects tested.

15857

Comparative Inhibitory Effect of Penicillin and Streptomycin Upon the Action of Staphylocoagulase.*

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One of the properties of pathogenic staphylococci is the capacity to induce the formation of a fibrin clot when the organisms are added to citrated human plasma. Though the significance of this biologic phenomenon is not clear, Hale and Smith[1] have pointed out that the *in vitro* phagocytosis of coagulase-positive staphylococci is inhibited in the presence of coagulable plasma and suggested that the invasiveness of these strains is associated with the *in vivo* production of a coagulum around the cocci which prevents efficient phagocytosis and destruction of the bacteria. Spink and Vivino[2] showed that the sulfonamides had a variable effect on the action of staphylocoagulase.

The present study revealed that streptomycin had a marked inhibitory effect on the action of staphylocoagulase in contrast to the results obtained with penicillin.

Material and Methods. Eighteen strains of coagulase-positive staphylococci were utilized; 5 strains being stock cultures, and 13 of them recently isolated from the lesions of patients. The strains were grown for 24 hours in tryptose phosphate broth and then 1 ml of each culture was added to a series of 11 sterile test tubes. Serial dilutions of penicillin and streptomycin were freshly prepared in physiologic saline solution and 1 ml amounts of the desired dilutions were added to each of the tubes containing organisms. The initial concentrations per ml of each antibiotic were 50,000 units, or 15 mg of crystalline penicillin G sodium, and 250 mg of streptomycin (base equivalent). The coagulase tests were performed by adding 0.2 ml of each culture-antibiotic mixture to 0.5 ml of citrated human plasma contained in small test tubes. The tubes were placed in a water bath at 37°C and examined for the presence of a coagulum after 2, 12 and 24 hours. Coagulation was graded from 1+ to 4+, 1+ indicating a coagulum that was just visible, while 4+ meant the formation of a solid clot, and 2+ and 3+ were designated as intermediate coagulation. The contents of each tube of antibiotic-culture mixture were tested for the presence of viable cells by streaking standard loopful amounts on agar plates, and incubating at 37°C for 24 hours.

* Aided by grants from Sharp and Dohme, Inc., and the Graduate School, University of Minnesota.

1 Hale, J. H., and Smith, W., *Brit. J. Exp. Path.*, 1945, **26**, 209.

2 Spink, W. W., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 37.

TABLE I.

Results with Strain 19-11 in Which Tests for Activity of Staphylocoagulase and Growth Inhibition Were Made Immediately After Mixing Culture with Penicillin or with Streptomycin.

Penicillin						Streptomycin				
Concentration per ml		Action of staphylocoagulase			Growth		Action of staphylocoagulase			Growth
Mg	Units	2 hr	12 hr	24 hr	24 hr	Mg per ml	2 hr	12 hr	24 hr	24 hr
15.	25,000	0	0	±	0	125.	0	0	0	0
7.5	12,500	±	1+	1+	0	63.	0	0	0	0
3.75	6,250	1+	2+	2+	0	31.	0	0	0	0
1.87	3,125	1+	2+	2+	0	16.	0	0	0	0
.93	1,562	1+	2+	3+	+	8.	0	0	0	0
.47	780	1+	2+	3+	+	4.	0	0	0	+
.23	390	1+	3+	3+	+	2.	0	0	0	+
.12	195	1+	3+	2+	+	1.	0	0	0	+
.06	98	2+	3+	2+	+	0.5	0	0	0	+
.03	49	2+	3+	2+	+	0.25	0	0	0	+
Control	0	3+	3+	2+	+	0	0	0	0	+

Two procedures were followed in detecting the effect of the antibiotics on the action of staphylocoagulase. In procedure A, the coagulase and viability tests were set up immediately after the antibiotics and organisms had been mixed. In procedure B, mixtures of antibiotics and organisms were incubated for 24 hours at 37°C before carrying out the coagulase and viability tests. Comparative studies were made with the 2 procedures to determine whether the length of time the bacterial cells were exposed to the antibiotics affected the action of staphylocoagulase. In each instance, control tubes containing only staphylococci and physiologic saline solution were used.

Results. Table I presents the data on one experiment with strain 19-11 in which procedure A was followed. It is to be noted that streptomycin markedly inhibited the action of staphylocoagulase even in the presence of viable cells in contrast to the inhibition produced by penicillin. Table II expresses the results of an experiment with the same strain using procedure B. The results are comparable to those given in Table I. It was observed that the incubation of the cells and antibiotics for 24 hours, as carried out in procedure B, delayed the clotting effect of staphylocoagulase longer than that obtained with procedure A in which no preliminary period of incubation took place before testing for coagulase activity. But this retardation of the action of coagulase was not associated with an increased destruction of organisms.

Similar results were obtained with the remaining 17 strains. It is of interest that low concentrations of streptomycin failed to inhibit the coagulase activity of only one of the 18 strains (strain 6-16). This strain proved to be moderately resistant to the lethal action of streptomycin. Relative resistance to penicillin was also shown with 3 strains, but it is to be pointed out that a large inoculum of organisms was used throughout the experiments. The inhibition of staphylocoagulase activity did not parallel the complete inhibition of growth by streptomycin. In other words, the activity of staphylocoagulase was completely inhibited by much lower concentrations than that required to render the cultures nonviable. The results with streptomycin are in contrast with those obtained with penicillin in which the inhibitory effect was less pronounced. Table III is a summary of the comparative results of coagulase activity in the presence of streptomycin and penicillin, after the mixtures of antibiotics and organisms had been incubated for 24 hours. There is a statistically significant difference between the degree of inhibition of coagulase activity as exhibited both by streptomycin and penicillin in the lower concentrations. Thus, while penicillin exerted some inhibitory effect on the activity of staphylocoagulase in the higher concentrations, its effectiveness was considerably diminished and

TABLE II.

Results with Strain 19-11 in Which Tests for Activity of Staphylocoagulase and Growth Inhibition Were After Mixtures of Culture and Penicillin or Streptomycin Had Been Incubated for 24 Hours at 37°C.

Penicillin						Streptomycin				
Concentration per ml		Action of staphylocoagulase			Growth		Action of staphylocoagulase			Growth
Mg	Units	2 hr	12 hr	24 hr	24 hr	Mg per ml	2 hr	12 hr	24 hr	24 hr
15.	25,000	0	0	0	0	125.	0	0	0	0
7.5	12,500	0	0	0	0	63.	0	0	0	0
3.75	6,250	0	0	0	0	31.	0	0	0	0
1.87	3,125	0	0	0	0	16.	0	0	0	0
.93	1,562	0+	1+	±	+	8.	0	0	0	0
.47	780	1+	2+	2+	+	4.	0	0	0	0
.23	390	1+	3+	3+	+	2.	0	0	0	+
.12	195	1+	3+	3+	+	1.	0	0	0	+
.06	98	2+	3+	3+	+	0.5	0	0	0	+
.03	49	2+	3+	3+	+	0.25	0	0	0	+
Control	0	1+	2+	1+	+	0				

TABLE III.

Summary of Comparative Inhibition of Staphylocoagulase Activity of 18 Strains by Varying Concentrations of Penicillin and Streptomycin.

Pencillin				Streptomycin			
Concentration, mg/ml	Inhibition	No. of strains	%	Concentration mg/ml	Inhibition	No. of strains	%
15.	None	1	5.55	16. -31	Slight	3	16.65
3.75-14.99	Slight	12	66.25	4. -8	Moderate	1	5.55
.23- 3.74	Moderate	4	22.22	.25- 2	Marked	5	27.77
.03- .22	Marked	1	5.55	<.25	Very marked	9	50.00

lost with lower concentrations while the inhibitory effect of streptomycin was pronounced even in concentrations of 0.25 mg per ml. The comparative results with the 2 antibiotics have been expressed quantitatively with respect to the actual weights of penicillin and streptomycin in each millimeter of mixture. It should be pointed out that streptomycin has a higher molecular weight than crystalline penicillin G sodium, but when comparison is made with the molecular equivalents of each of the antibiotics, streptomycin still has a more marked effect on the action of staphylocoagulase than penicillin.

The mechanism whereby penicillin, and to a greater extent, streptomycin inhibit the action of staphylocoagulase requires further study. The foregoing results show that inhibition by streptomycin occurred in the presence of viable cells. However, this does not necessarily imply that the antibiotic acted directly upon staphylocoagulase or blocked the reaction between staphylocoagulase and plasma.

Summary. Comparative studies with penicillin and streptomycin on 18 strains of coagulase-positive staphylococci revealed that the action of staphylocoagulase on human plasma is inhibited to a greater extent by streptomycin than by penicillin.

15858

A Simple Means to Determine Exact Moment of Clotting in Prothrombin or Thrombin Time Determination.

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The determination of the exact moment of coagulation by direct visual observation is at times difficult, especially if the solutions are water clear as happens for instance in following the action of purified thrombin on a fibrinogen solution. By employing the principle that the fluid in a test tube remains stationary when the tube is slowly revolved, one can accurately time the beginning of solidification since at that moment the contents revolve with the tube. The procedure as applied to the prothrombin time (one-stage method) is as follows: 0.1 cc of oxalated plasma is transferred to a small pyrex test tube (100 x 13 mm) by means of a 1 cc pipette graduated in 0.1 cc cut to 170 mm length (as recommended by Quick[1] for his method) and fitted with a rubber bulb from a medicine dropper. In transferring the plasma the tip of the pipette must touch the bottom of the tube and enough air blown through to produce a few bubbles which gather about the periphery. At least one-half of the circumference should be free of bubbles. The thromboplastin (0.1 cc) is carefully added, and then 0.1 cc of 0.02 M $CaCl_2$ is forcefully blown in to assure prompt mixing. At this moment the stop watch is started. The upper part of the tube is held by the last 2 fingers of the right hand and rotated slowly with the thumb and the first finger. By practice one learns the most advantageous angle at which the test tube is to be held and likewise the best speed of rotation. The bubbles are closely watched and the moment they revolve with the tube, the stop watch is clicked.

The test is performed in a glass water bath (the type used for the Wassermann reaction) kept at 37°C and illuminated by a desk lamp placed opposite the observer. The light must be focused so that the contents of the test tube can be observed while immersed in the bath. All reagents as well as the test tubes used for the determination are kept in the water bath.

[1] Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 211.

15859

Intravenous Carbohydrate Tolerance Tests on Swine.

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Early work[1–4] on carbohydrate tolerance indicated that the rise and fall of blood sugar after the administration of glucose are rapid and reproducible tests of the regulating mechanisms of carbohydrate metabolism. Although the reliability of the method as a diagnostic tool has been questioned because of divergent results obtained after repeated tests on the same normal individual, the procedure is of distinct value if the test is car-

[1] Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, **1**, 299.

[2] Gilbert, A., and Baudouin, A., *C. R. Soc. biol.*, 1908, **65**, 710.

[3] Bang, I., *Biochem. Z.*, 1913, **49**, 19.

[4] Jacobsen, A. Th. B., *Biochem. Z.*, 1913, **56**, 471.

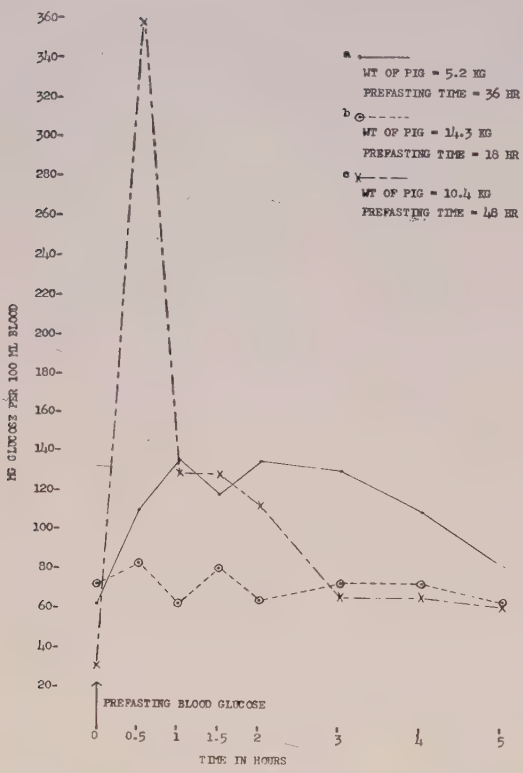

FIG. 1

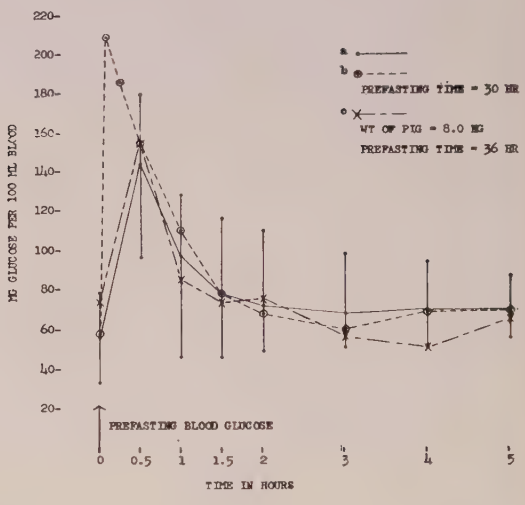

FIG. 2

FIG. 1. a. Oral carbohydrate tolerance test on a pig given 2 g glucose per kilo body weight by stomach tube. b. Intravenous carbohydrate tolerance test on a pig following a prefasting period of 18 hr. c. Intravenous carbohydrate tolerance test on a pig following a prefasting period of 48 hours.

FIG. 2. a. Average of 17 intravenous carbohydrate tolerance tests on pigs fasted 24 to 36 hr prior to injection of glucose. Vertical lines through each point indicate deviations from the mean. b. Average of 3 intravenous carbohydrate tolerance tests on pigs bled 5 and 15 minutes after injection of glucose. c. Intravenous carbohydrate tolerance curve on pig showing fall in blood glucose below prefasting level.

ried out by experienced investigators and the results evaluated critically.[5]

Carlson and Drennan[6] reported that "the normal pig has a lower tolerance for dextrose, bread or cooked starch given by mouth than any species so far studied." This is contrary to what would be expected because pigs that are full-fed presumably are able to digest and assimilate high carbohydrate diets. Inasmuch as corn, a cereal which contains approximately 70% starch, is usually the chief constituent in rations fed to swine[7] and since only limited data are available regarding carbohydrate metabolism in the pig, further study of carbohydrate tolerance in this species seems advisable.

Methods. In the study reported herein oral carbohydrate tolerance tests were made on 20 healthy weanling pigs. Two grams of glucose per kg of body weight, in 50% solution, were administered by stomach tube. The pigs were fasted for periods of 24, 36, 48 and 96 hours prior to giving the test dose, but in no instance was the typical carbohydrate tolerance curve of the normal human obtained. (Fig. 1a). Since humans and

5 Bodansky, M., and Bodansky, O., *Biochemistry of Disease,* The Macmillan Company, New York, 1940.

6 Carlson, A. J., and Drennan, F. M., *J. Biol. Chem.*, 1912-1913, **13**, 465.

7 Morrison, F. B., *Feeds and Feeding,* 20th Ed., The Morrison Publishing Company, Ithaca, N.Y., 1942.

swine are omnivorous and have simple stomachs, one might expect each of these species to respond in similar fashion to ingestion of glucose. Nevertheless, the results obtained seemed to indicate that this method is unsatisfactory in swine probably because, as pointed out by Dukes,[8] the gastric emptying time is prolonged in the pig. In order to diminish this effect we turned to the intravenous route of administering glucose.

The blood sugar concentration apparently can be raised to almost any level when concentrated solutions of glucose are injected intravenously, but the disposal and subsequent reactions are presumably comparable to those elicited when glucose is given orally.[9] Two preliminary tests suggested that a prefasting period of 18 hours was hardly adequate, since the tolerance curves were irregular with a lag in the rise of the sugar level (Fig. 1b). If, on the other hand, the pigs were fasted 24 hours, a satisfactory curve was obtained, but the blood glucose concentration at the 30-minute interval was not more than 15 to 20 mg higher than the value at the prefasting level. Thus, to increase this span it was necessary to fast the pigs at least 30 hours but not over 36 hours. If the fast was prolonged beyond 36 hours, an exaggerated hyperglycemic response resulted which is the usual consequence of prolonged fasting[5] (Fig. 1c).

Seventeen intravenous carbohydrate tolerance tests were made on healthy pigs ranging in weight from 5.9 kg to 30.8 kg and averaging 11.4 kg. A prefasting sample of blood for glucose determination was withdrawn from the anterior vena cava by the technic of Carl and Dewhirst.[10] Seventy-five hundredths of a gram of glucose per kg of body weight was administered intravenously in 50% solution at a rate of approximately 0.3 g per kg per minute. The concentration of blood glucose was determined by the Shaffer, Hartmann, Somogyi method as described by Koch.[11] Throughout each test the pigs were handled carefully and bled rapidly to eliminate the factor of undue excitement, which in turn might produce a rise in blood glucose.

Average values of blood glucose for 17 healthy pigs during each half-hour interval of the intravenous carbohydrate tolerance test are shown in Fig. 2a. There was a wide variation in the prefasting blood glucose concentration with the levels ranging from 33.90 mg % to 79.10 mg % and an average of 56.62 mg. This divergence may be attributed in part to the age of the pigs and the period of inanition prior to the test. Five minutes after injecting glucose the average concentration on 3 pigs was 209.46 mg glucose per 100 ml blood, whereas in 15 minutes the value dropped to 186.07 mg % (Fig. 2b). Likewise, there was a marked divergence in concentration of blood glucose at the half-hour interval, varying from 97.18 mg % to 179.74 mg % with an average of 144.55 mg per 100 ml blood. During the next half-hour the average drop in glucose concentration was 46.44 mg %, suggesting rapid oxidation and utilization of carbohydrates by the pig. At 1½ and at 2 hours the concentrations fell to an average of 78.96 and 72.99 mg % respectively. In 2 hours, for 6 individual tests, the concentration of glucose dropped to a value below the prefasting level. A similar decline is often observed in oral tests on humans,[5] with the glucose value rising to the prefasting level or slightly above toward the end of the trial (Fig. 2c).

In 5 of the trials, urine was collected during the test and only relatively small amounts of sugar were recovered varying from 0.15 to 0.88 g with an average of 0.5 g representing about 5% of the total quantity injected. This is comparable to the amount of glucose recovered in the urine of the bovine following intravenous administration of glucose during carbohydrate tolerance tests in this

8 Dukes, H. H., *The Physiology of Domestic Animals,* 5th Ed., Comstock Publishing Co., Inc., Ithaca, N.Y., 1942.

9 Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry. Interpretations,* Vol. I, 2nd Ed., The Williams and Wilkins Company, Baltimore, 1946.

10 Carle, B. N., and Dewhirst, Wm. H., Jr., *J. Am. Vet. Med. Assn.,* 1942, **101**, 495.

11 Koch, F. C., *Practical Methods in Biochemistry,* 3rd Ed., The Williams and Wilkins Company, Baltimore, 1941.

species.[12] It appears, therefore, that intravenous carbohydrate tolerance curves obtained on healthy pigs are quite similar to those procured on normal human subjects.

12 Bell, F. R., and Jones, E. R., *J. Comp. Path.*, 1945, **55**, 117.

Summary. The form of the carbohydrate tolerance curve obtained after the administration of glucose to normal growing pigs by the intravenous route, apparently closely parallels the type of curve procured when the test is made on normal human subjects.

15860

Thromboplastic Activity of the Urine.

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Hemophilics having hematuria often experience severe attacks of renal colic and pass thin clots obviously representing ureteral casts. Since the clotting time of the blood of one such patient observed in January 1947 was in excess of 2 hours, the formation of such clots within the ureter was difficult to explain, unless blood stagnation existed because of obstructive lesions, or the urine itself exerted a clot accelerating action on the blood. The presence of thromboplastic substances in the tissues of the body, in the saliva from normal[1,2] and hemophilic men[3] and in human milk[4] is known. No mention of the presence of these substances in the urine has been found, and there seems to be no adequate explanation of the mode of formation of clots in the renal pelvis of hemophilics with hematuria.[5–10] The evidence to be presented indicates that urine from normal or hemophilic men contains a thromboplastic substance. Contact with such a substance is probably what causes clotting of the blood extravasated in the renal pelvis.[11]

Testing of clot accelerating activity was done in most instances by adding 0.1 cc of the material to be tested to 1 cc of normal or hemophilic venous blood collected with oiled syringes and kept in paraffin-coated tubes. Unless otherwise stated, collodion-coated glass tubes (13 mm internal diameter) were used for the clotting time determinations. The urine was collected, usually from males, over a period of 24 hours in clean jugs containing a crystal of thymol; it was filtered or centrifuged before use. The usual chemical and microscopical studies were carried out on each urine. Those specimens containing sugar, albumin, casts, blood cells or bacteria were not used.

1. The simple addition of intact urine to normal or hemophilic blood accelerates its coagulation in glass or collodion-coated tubes (Table I). Dilution of the urine reduces its clot accelerating ability. Urine obtained by catheterization from the bladder or the renal pelvis does not differ significantly in its ac-

1 Bellis, C. J., and Scott, F. H., PROC. SOC. EXP. BIOL. AND MED., 1932, **30**, 1373.

2 Glazko, A. J., and Greenberg, D., *Am. J. Physiol.*, 1939, **125**, 108.

3 Tocantins, L. M., unpublished observations.

4 Jacoby, M., and Adler, S., *Enzymologia*, 1937, **1**, 373.

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6 Schlossmann, H., *Die Hämophilie*, Enke, 1930.

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8 Quick, A. J., *Hemorrhagic Diseases*, Thomas, 1942.

9 Nygaard, K. K., *Hemorrhagic Diseases*, Mosby, 1941; Ferguson, J. H., *Ann. Rev. Physiol.*, 1946, **8**, 231.

10 Fonio, A., *Ergeb. der Inner. Med. u. Kinder heil.*, 1936, **51**, 443.

11 Tocantins, L. M., and Lindquist, J. N., *Fed. Proc.*, 1947, **6**, 215.

TABLE I.

Rate of Coagulation of Normal and Hemophilic Venous Blood to Which Were Added Variously Collected and Filtered Normal or Hemophilic Urine (0.1 cc urine, 1 cc blood in collodion coated tubes at 38°C). Clotting time in seconds.

	Source of urine																
	Normal Man*						Hemophilic Man*						Normal Woman†				Control
	Filtration						Filtration										
	None		Paper†		Berkef.‡		None		Paper†		Berkef.‡		Bladder		Right Renal Pelvis	Left Renal Pelvis	0.85% NaCl
Dilution:	0	1-5	0	1-5	0	1-5	0	1-5	0	1-5	0	1-5	0	1-5			
Normal blood	438	642	477	808	510	705	514	794	600	792	492	696	394	642	368	373	898
Hemophilic blood	530	812	461	811	1414	2914	506	795	671	786	1493	2878	670	1760	537	562	>7200

* Urine voided spontaneously. Tests for protein and glucose negative. Microscopic examination negative.
† Urine filtered through double thickness Whatman No. 2 paper.
‡ Urine filtered once through a Berkefeld V candle.

TABLE II.

Physiochemical Changes in Urine After Dialysis; Effect of Addition of Fresh and Dialysed Urine on Rate of Coagulation of Normal and Hemophilic Blood.

Type of urine	Spec. grav.	pH	Total nitrogen (g %)	Chlorides (g %)	Sulfates (as SO_3) (mg %)	Inorganic phosphates (mg %)	Clotting time (sec)‡	
							Normal blood	Hemophilic blood
Fresh								
Undilut.	1020	5.35	3.32	1.0	250	300	530	506
Dilut. 1-5							812	795
Dialysed*								
Undilut.	1000	6.5	0.005	0	117.4	1.8	275	261
Dilut. 1-5							518	720
Controls								
1. 0.85% NaCl							898	>5600
2. Dialysed H_2O alone†							—	>5600

* Salt content adjusted by adding NaCl to 0.85% concentration before mixing with blood.
† Distilled water "dialysed" against running water for 48 hours.
‡ 1 cc blood, 0.1 cc solution in collodion coated tubes at 38°C.

TABLE III.
Effect of Heating (15 min) Undialysed and Dialysed Urine on Its Thromboplastic Activity Tested on Hemophilic Blood.*

	Unheated urine		Urine heated 55°C		70°C		100°C	
Dilution:	0	1-5	0	1-5	0	1-5	0	1-5
1 cc blood, 0.1 cc intact urine (sec)	401	666	887	775	2950	2108	7087	3050
1 cc blood, 0.1 cc dialysed† urine (sec)	337	563	518	712	599	1009	763	1482

* Clotting time in collodion tubes $>$ 7200 sec.
† Dialysis in "Visking" casings for 48 hr against cold running water.

TABLE IV.
Effect of Heating Urine to 70°C (15 min) Before and After Dialysis on Its Thromboplastic Activity Tested on Hemophilic Blood.

	Fresh urine*		Urine heated at 70°C only		Urine dialysed only		Urine heated at 70°C then dialysed		Urine dialysed, then heated at 70°C	
Dilution:	0	1-5	0	1-5	0	1-5	0	1-5	0	1-5
1 cc blood, 0.1 cc urine, clotting time (sec)	401	666	2950	2108	337	567	4400	6035	599	1009

* Filtered through double thickness Whatman No. 2 paper.

tion from urine voided spontaneously. Urine collected from hemophilics behaves essentially like that collected from normal men. The activity is not altered by paper filtration (or centrifugation), but passing the urine through a Berkefeld V candle reduces its clot acceleration power, evident only when the testing is done on hemophilic blood (Table I). The clot accelerating effect is not due to the addition of prothrombin since a mixture of brain thromboplastin, $CaCl_2$, the urine or its dialysed dried residue, and prothrombin-free fibrinogen will not lead to clotting. It is not due to thrombin, since direct addition of intact or dialysed urine to a fibrinogen solution will not change it to fibrin. Moreover, when the urine or its dialysed dried residue are added to unrecalcified citrated plasma no clot forms, after standing 24 hours. The clot accelerating activity of urine and its extracts is apparently due, therefore, to the presence of a thromboplastin-like activity.

2. When the clear protein-free urine is dialysed in cellophane bags for 48 hours against cold running water, most of its electrolytes and nitrogen containing compounds are removed and the clot accelerating power of the urine is somewhat increased (Table II). The effect of heating is more pronounced on intact than on dialysed urine (Table III). The heated undialysed urine has a greater clot accelerating power when diluted, an indication that in fresh urine, inhibitors are present which reduce the effectiveness of the clot accelerators. If the urine, however, is dialysed after being heated, the relatively greater clot accelerating effect of the diluted urine is no longer observed (Table IV). Dialysis may remove a substance which has an anticoagulant action of its own; when the intact urine is only heated, the reduced activity of the urine thromboplastin may allow the action of a heat-resisting anticoagulant to assert itself.

3. Clear, filtered dialysed urine may be frozen and its water content removed by the lyophil method. A fine light brown powder results. The average yield of 10 lots was 43.4 mg of solids per 100 ml of dialysed urine (range 72.-29.5 mg). In concentrations above 100 mg %, the material is poorly soluble.

The addition, *in vitro,* to normal and hemophilic blood, of concentrated solutions of the dried residue has a pronounced clot accelerating action. With the stronger solu-

TABLE V.
Effect of Addition of Dialysed Urine Residue on Rate of Coagulation of Normal and Hemophilic Blood in Collodion and Glass Tubes.

	Mg of lyophilized dialysed urine residue added to each ml blood							
	1.0	0.5	0.1	0.05	0.01	0.005	0.001	Control 0.85% NaCl
Hemoph. blood*								
Collod. tube (sec)	244	282	307	484	1550	2566	4466	>7200
Glass tube (sec)	234	242	340	375	725	998	2600	4300
Normal blood*								
Collod. tube (sec)	230	220	250	289	498	595	878	1652
Glass tube (sec)	229	252	280	332	514	554	781	936

* 1 cc blood, 0.1 cc sol. of residue in 0.85% NaCl.

TABLE VI.
Diminution in Thromboplastic Activity of Urine Standing at 5°C for Several Days.

	Days after collection						
	1	3	4	12	16	27	38
	Clotting time in sec.						
Hemophilic urine*	184	253	265		589	966	903
Normal urine*	234	278	227		210	210	365
" " *	245			795		1180	1167

* 0.1 cc urine, 1 cc hemophilic blood (clot. time >7200") in collodion coated tubes at 38°C.

tion the intensity of the effect is about the same, whether glass or collodion tubes are used; with weaker solutions the thromboplastic effect is less marked on hemophilic blood in collodion tubes (Table V). The coagulation was not reduced below 200 seconds, even when the concentration of the powdered material in the solution added was increased so that 3. mg were added to each ml of blood.

4. Standing at 5°C of intact or dialysed urine leads to slow diminution of its clot accelerating activity (Table VI). The activity of the dried material is likewise reduced on standing, even when kept in evacuated ampuls. Dialysed urine appears to maintain its activity longer than intact urine. Mixing the lyophilized residue with ethyl ether or absolute ethyl alcohol (100 cc per g of residue) for 2 hours does not seem to affect the clot accelerating activity of the residue. Advantage was taken of this fact to render the preparations bacteriologically sterile.

5. A single or repeated intravenous injection of the dialysed lyophilized material (60 mg/kg body weight) into rabbits at a rapid rate (20 mg per second), has a temporary prostating effect lasting about 5 minutes, from which the animal recovers without any subsequent deleterious effects. Intravenous injection into anesthetized dogs of the same material in amounts of 2.5 mg per kg body weight, over a period of 60 seconds, has a moderate transitory depressing effect on blood pressure, from which the animal usually recovers within 10 minutes.

Intravenous infusion of a solution of the lyophilized residue into a hemophilic, in doses indicated on Chart I, leads to a diminution in the rate of blood coagulation in both glass and collodion tubes, the intensity and duration of the response varying according to the amount of material injected. There are no symptoms during the infusion when the rate is maintained between 5-10 mg per minute. When this is exceeded, flushing, pounding behind the eyes and in the abdomen and a slight headache is felt. In 2 instances when the dose of material was 3-4 mg per kg body weight, slight fever, malaise, joint pains and diuresis occurred for a period of 24 hours following the infusion.

Comment. Both intact urine and that free of most of its solutes by prolonged dialysis seem to contain a substance capable of accelerating the coagulation of normal and

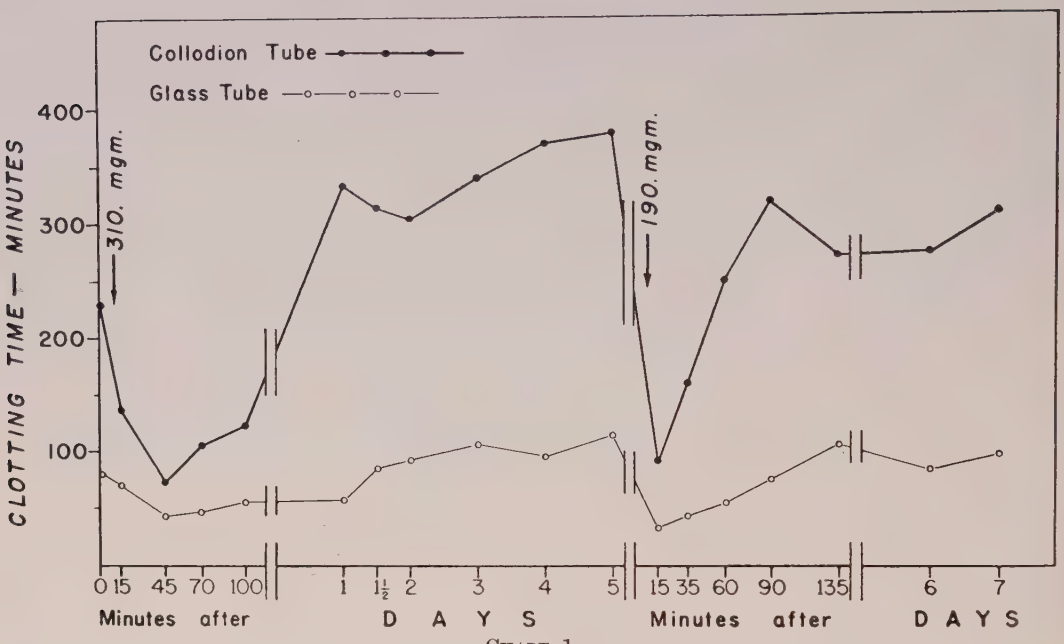

CHART 1.

Effect of 2 intravenous infusions of solutions of the lyophilized dialysed urine residue on the clotting time of the blood of a hemophilic.

hemophilic blood *in vitro* and *in vivo*. The clot accelerating substance in the urine behaves like a thromboplastin and may be derived from one of the cellular or chemical components of the urine, the kidney itself, or represent thromboplastic material separated from the blood and cleared through the kidney in the urine. If the latter proves to be the principal source of the urinary thromboplastin, it is difficult to understand how hemophilic urine often displays as much and occasionally greater activity than normal urine, since hemophilic plasma is said to contain less thromboplastin than normal plasma.

Undialysed and dialysed urine are known to contain blood pressure depressors which apparently differ in action from histamine.[12] A fraction has been separated[12] which has a pronounced blood pressure depressing effect. The substance responsible for this effect is apparently nondialysable, since it may be found in the dialysed urine or a solution of its lyophilized residue. If the blood pressure depressing fraction proves to differ from that having the clot accelerating effect, it may be possible to separate the 2, and to use the thromboplastic fraction for the temporary correction of the coagulation defect in hemophilia. By supplying an excess of thromboplastic activity, the excess of anticephalin activity in hemophilic blood[13] may be temporarily offset, and the coagulation of blood made to approach normal levels.

The urine thromboplastin behaves toward heat somewhat like aqueous solutions of brain thromboplastin. The latter when heated to 100°C lose much of their activity. The potency and heat stability of thromboplastic solutions are known to be influenced by the presence of other substances in the solution. This may explain why the thromboplastin of the dialysed urine is more thermoresistant than that of fresh urine. It is also possible—and the dilution experiments so indicate—that fresh urine contains clot inhibiting substances as well, which are more resistant to heat than the thromboplastin. These substances seem to be lost during dialysis, thus

[12] Frey, E. K., and Kraut, H., *Arch. f. Exp. Path. u. Pharmac.*, 1928, **133**, 1; Westerfeld, W. W., and coworkers, *Am. J. Physiol.*, 1944, **142**, 519.

[13] Tocantins, L. M., *Blood*, 1946, **1**, 156.

allowing the effect of the thromboplastin to become the dominant one in the dialysed urine.

Summary. The clear protein and cell-free urine from normal men and women and from hemophilic men has thromboplastic activity when tested on normal and hemophilic blood. The thromboplastin in dialysed and lyophilized urine seems more resistant to heat than that in fresh urine. The lyophilized dialysed urine residue has a clot accelerating action on hemophilic blood *in vitro* and *in vivo*. The clots which form in the ureter of hemophilics with hematuria are probably due to contact of the extravasated blood with the thromboplastin of the urine.

15861 P

"Control" Erythrocytes for Hemolysis Studies.*

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In several previous experiments the permeability of chicken erythrocytes at 37.5°C was studied over periods of many hours' duration. It was noted that the time for hemolysis in glycerol of untreated cells became less after several hours (Hunter).[1] Since no attempt was made in these experiments to control bacterial contamination, it was thought that a part of the change in these control cells might have resulted from bacterial action. In another series of experiments, however, in which aseptic technics were followed it was also observed that the time for hemolysis of control cells decreased with lapse of time (Hunter and Larsh).[2] Results of this nature might have been predicted in view of previous experiments. Jacobs and Parpart[3] have shown that erythrocytes are quite sensitive to environmental changes while Harris[4] showed that under certain conditions of standing, erythrocytes lose potassium.

First a series of experiments were performed in which blood was allowed to stand at 37.5°C and time for hemolysis in glycerol was measured at varying intervals of time. Aseptic technic was observed throughout and tests were made for bacterial contamination as before (Hunter and Larsh).[2] Four stock suspensions were made. (1) Two cc of freshly drawn, heparinized chicken blood, (2) 1 cc of blood plus 1 cc of Ringer Locke, (3) 1 cc of cells plus 1 cc of plasma and (4) 1 cc of cells plus 1 cc of Ringer Locke. The results obtained using whole blood are shown in Fig. 1. Essentially similar results were obtained with all 4 solutions except the Ringer Locke appeared to exert a "protective effect" especially in (4). A similar series was run using blood which had been in the refrigerator for 27 hours. Hematocrit readings indicated that these cells had swollen although the initial hemolysis times and subsequent

TABLE I.
Effect of Standing on Hemolysis Time and Volume.

Time of exposure in hr	Time in sec for 50% hemolysis	Cell volume in μ^3
0	285	90
12⅓	245	104
0	395	112
23½	200	123
0	300	118
29¾	160	138

* The authors are indebted to the Faculty Research Fund of the University of Oklahoma for grants in aid.

1 Hunter, F. R., *J. Cell. and Comp. Physiol.*, 1947, in press.

2 Hunter, F. R., and Larsh, Howard W., to be published.

3 Jacobs, M. H., and Parpart, A. K., *Biol. Bull.*, 1931, **60**, 95.

4 Harris, J. E., *J. Biol. Chem.*, 1941, **141**, 579.

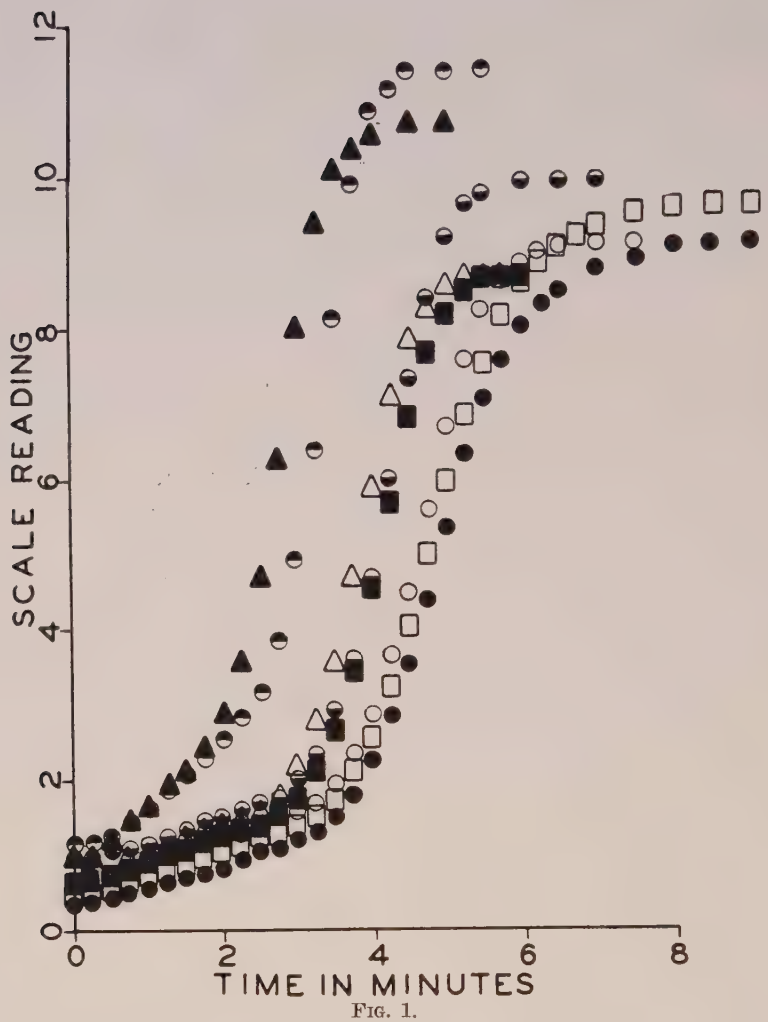

FIG. 1.

Effect of time on the hemolysis of chicken erythrocytes in glycerol. □—0 hr; ●—¾ hr; ○—3½ hr; ■—7½ hr; △—10¼ hr; circle with solid bottom—12¾ hr; circle with solid top—28¾ hr; ▲—51¾ hr.

changes paralleled those obtained with the freshly drawn blood.

A second series of experiments involved hemolysis measurements, cell counts (Parpart),[5] and hematocrit determinations using an air turbine. These data are presented in Table I. It can be seen that with the passage of time at 37.5°C the cells swell as well as hemolyze more rapidly.

To determine whether the swelling could account for the change in rate of hemolysis, equal volumes of freshly drawn blood and 80% Ringer Locke (80 cc of Ringer Locke plus 20 cc of water) were mixed. At the end of 10 minutes the measurements were made. These data are presented in Table II.

As expected, the cells in the 80% Ringer Locke swelled but the hemolysis time at 0 hours was unaffected. After a lapse of 20-24 hours, these cells increased in volume but to a lesser extent than the untreated cells. The change in hemolysis times of these treated cells was less than for the untreated.

The fact that erythrocytes in whole blood maintained at 37.5°C under aseptic condi-

[5] Parpart, A. K., *Biol. Bull.*, 1931, **61**, 500.

TABLE II.
Effect of Initial Swelling on Hemolysis Time.

Time of exposure in hr	Time in seconds for 50% hemolysis		Cell volume in μ^3	
	Plasma	80% R.L.	Plasma	80% R.L.
0	345	350	112	128
20	200	230	126	139
0	365	370	123	132
23¼	190	220	136	139
0	340	340	125	143
24	205	240	146	160

tions change so markedly indicates additional factors which must be taken into consideration in hemolysis experiments. No satisfactory explanation is available at present for the apparent lack of effect of a slightly hypotonic environment on hemolysis time.

In summary it may be said that control chicken erythrocytes after standing at 37.5°C for several hours hemolyze more rapidly. An increase in the volume of these cells was observed but this cannot be the sole explanation for the change in rate of hemolysis.

15862

A Simple Inexpensive Pump for Perfusion of Organs with Preservatives or with Physiological Solutions.*

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In an attempt to investigate the secretory properties of human testes by means of perfusion *in vitro*, 3 types of perfusion pumps were employed but none were found to be suitable for these purposes. The Carrel-Lindbergh pump[1] proved to be so readily subject to disturbances upon change in the resistance offered by the organs during perfusion that maintenance of a proper flow of blood was difficult. The pump devised by Kleinberg and associates[2] is less complex, but in our experience the valves were prone to stick, and during long experiments trouble was encountered in stabilizing the flow of blood through the shunt and in preventing changes in the level of fluid in the compression chamber. The Fischer "Volustat"† came nearest to filling the requirements of our experiment but afforded no means for regulation of the rate of pulsation.

Study of the mechanical principles of these and other pumps, *e.g.*, the Dale and Schuster apparatus,[3] suggested the need for a pump with the following characteristics: (a) ready construction at small cost; (b) sufficient simplicity in operation as to provide rapid and satisfactory perfusion of an organ with physiological solutions or with preservative fluids; (c) no need of continuous adjustment during prolonged periods of perfusion; (d) the capacity to mimic the action of the heart by providing any desired rate of pulsation

* This study has been supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

1 Carrel, A., and Lindbergh, C., *The Culture of Organs*, Paul B. Hoeber, Inc., New York, 1938.

2 Kleinberg, W., Gordon, A., and Charipper, H., *J. Lab. Clin. Med.*, 1943, **28**, 1484.

† Fischer, "Volustat," Eimer and Amend catalogue 90, No. 13-684.

3 Dale, H., and Schuster, E., *J. Physiol.*, 1928, **64**, 356.

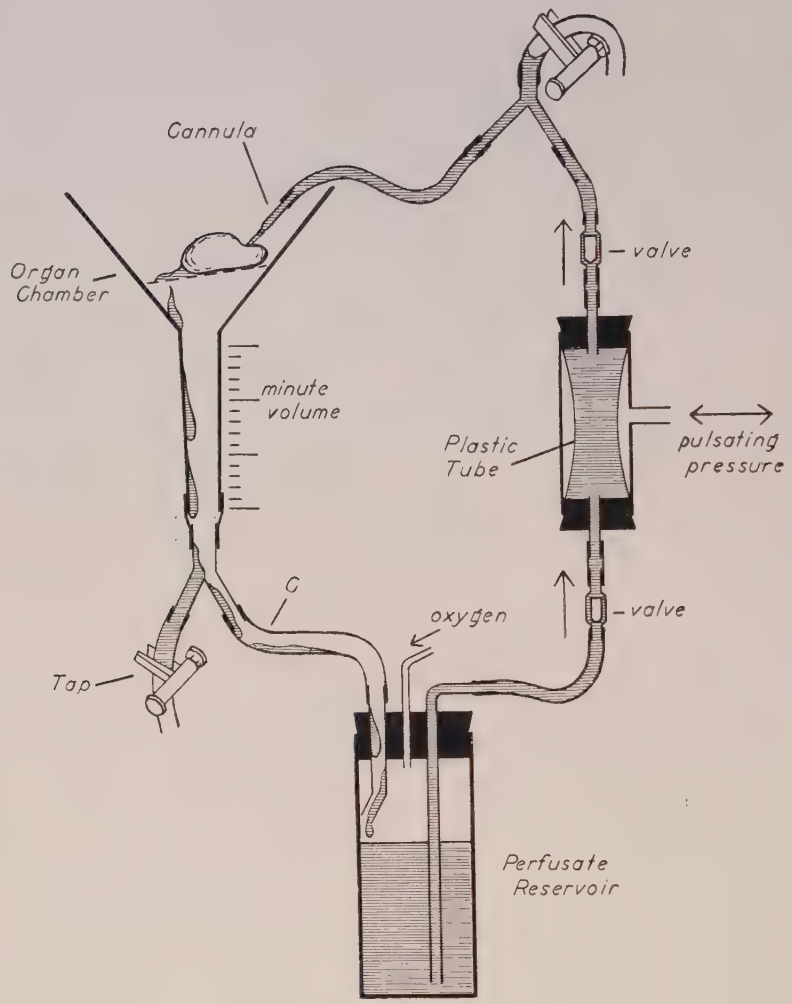

Fig. 1

and volume of fluid per pulsation; (e) allowance for the necessary degree of oxygenation of the perfusate; (f) the ability to use perfusates either in large quantities or amounts as small as 25 cc; (g) facilities for addition or removal of aliquots of the perfusate while the pump is in operation; (h) construction that permits easy determination of minute volume; (i) composition of materials that are easily sterilized and not toxic to tissues.

Description of Apparatus. The final design of the pump (Fig. 1) is the serial successor of many and was arrived at by a process of trial and error. Its essential parts are the organ chamber which is a funnel, the perfusate reservoir, the pump proper including its 2 valves, and tubing connecting these 3 parts.

The pump proper is a simple device made from a Pyrex test tube, 200 x 25 mm, with a side tube. The closed end of the test tube was removed. A piece of plastic tubing was placed inside the glass tube and pulled over each open end in such a way that when stoppers were inserted into the ends of these plastic and glass tubes the stretched plastic was held firmly in place. By interposing this between 2 valves directed as indicated in Fig. 1 and by supplying a pulsating pressure of air to the side tube, the pump becomes complete.

A positive thrust of air compresses the plastic tube and causes the lower valve to

close while the upper valve is opened; release of the positive thrust permits the elastic plastic tubing to expand and so causes the lower valve to open while the upper valve is closed. Fluid is thus pumped from the reservoir to the cannulated organ.

As stated above, the funnel represents the chamber in which the organ rests on a glass plate. From this the perfusate drips into the stem of the funnel. The inverted Y-tube attached to the stem makes it possible to deflect the perfusate into either the perfusate reservoir or the tube labelled "tap" in the illustration. With a clamp on the tap and a second clamp at the point "C" the minute volume can be estimated from the time required to fill the graduated funnel stem. Samples of perfusate may be obtained for analysis by removing the clamp from the tap.

The perfusate reservoir receives the fluid returning from the organ being perfused and also a tube bearing oxygen or any mixture of gases. If desired, the oxygen may be bubbled through the perfusate before it passes into the tube and up through the funnel stem to be dissipated. As the oxygen passes toward the organ chamber it contacts the descending film of perfusate.

From the perfusate reservoir the fluid is pumped into the organ thus completing the circuit.

Bubbles of gas may pass through the pump proper toward the cannula, when the fluid first flows through the apparatus or if the reservoir is agitated. An inverted Y-tube with a clamp guarding the outlet acts as an adequate trap to eliminate air emboli which might be pumped into the organ. The pressure in the system may also be measured at this site.

The pump receives a pulsating pressure of air, *i.e.*, a regular positive thrust alternating with reduction of pressure to that of the general atmosphere. Because a Carrel-Lindbergh apparatus was available we used the pulsating valve made at the Rockefeller Institute and driven by a 1/30 H.P. motor supplied by the Janette Manufacturing Company of Chicago. This motor is equipped with reduction gears and rheostat. Supplementing it one must have a source of compressed air. The pulsating valve and compressed air unit of the Carrel-Lindbergh apparatus are a luxury rather than a necessity. We have used successfully a heavy-walled rubber bulb compressed by a motor-driven adjustable plunger. The Wiggers' artificial circulation machine[4] used in many teaching laboratories has such a rubber bulb and works well with this apparatus.

The flexible plastic tube which receives the pulsating pressure has an inside diameter of 11/16 inch. The inside diameter of tubing used elsewhere was 5/32 inch except for the piece marked "C" in Fig. 1 which was 3/8 inch. The tubing is furnished by the Industrial Synthetics Corporation of 60 Woolsey Street, Irvington, N.J., under the tradename of Voltron.

The glass valves were obtained from an old-fashioned 2-way syringe used for blood transfusions. Glass ball-valves are also suitable. If the requirements of the perfusion are not too exacting, valves from a sphygmomanometer bulb may be employed.

Merits of the Apparatus. The apparatus can be assembled in less than one day's time at a cost of about 5 dollars. It is easy to operate. During perfusion of human testes and rat kidneys for as long as 8 hours minor adjustments were made only at intervals of an hour or more.

The stroke volume and rate of beating of the heart can be simulated over a wide range. Systolic pressure can be as high as 300 mm of mercury with a drop to zero in the diastolic phase when no organ is providing resistance. The number of pulsations per minute can be varied from 85 to 122 with the Carrel-Lindbergh pulsation valve.

While the perfusion is in progress aliquots of the perfusate may be removed or more added, thus allowing serial study of changes in the perfusate or trials of entirely different media. Pumps of various sizes might be employed. The present apparatus works with a volume as small as 25 cc but quanti-

[4] Wiggers, C. J., *Physiology in Health and Disease*, 4th edition, Lea and Febiger Co., Philadelphia, p. 643, 1944.

ties as small as 5 to 10 cc could probably be used if the apparatus were produced in miniature. This would permit perfusion of an organ of a small rodent with blood from the same animal.

The plastic tubing imparts mobility to the apparatus and reduces the danger of breakage. In addition this tubing is nontoxic for organs as judged by its effect upon tissue cultured in close association with it. To test this fragments of 8-day chick embryo heart ventricles were planted in equal parts of blood plasma and embryo extract. Before clotting occurred 3 chips of tubing were set close to each tissue fragment so that if growth occurred it would be over and around the plastic. Of several plastics tested, "Voltron" was the only one which had no apparent effects over an observation period of 3 days. Preliminary to cultivating the tubing was sterilized by immersion in 70% alcohol for 24 hours and the alcohol was removed by immersion in sterile Tyrode solution.

Limitations of the Apparatus. Among the more serious limitations of the apparatus is the fact that when whole blood is used in considerable quantity the corpuscles settle in the reservoir. A means for agitating the fluid in the reservoir would be a desirable addition to this apparatus.

For sterile procedures the funnel has to be replaced by a more elaborate organ chamber. Suitable chambers have a drainage tube and an entrance for a 2-hole stopper. One opening in the stopper admits the cannula, the other a cotton filter through which escapes the oxygen which passed to the organ chamber from the reservoir.

The apparatus contains no filters. For non-sterile work we set a small pad of cotton over the neck of the funnel. If needed, more elaborate sand filters can be employed.

Still other refinements might be adopted, but the pump has proved to be satisfactory as it is. For example, an entire rat can be rendered blue by fluids containing various amounts of trypan blue. As another illustration, microscopic examination of fresh spreads or sections of tissues from a rat perfused with Ranvier's gelatin carmine showed complete injection of the vascular trees in the intestines, kidneys, liver, and other organs. Many but not all of the vessels in the skeletal muscles appear to be filled.

Summary. Description is given of an easily constructed perfusion pump and of the satisfactory results obtained with its use.

A type of plastic tubing used in this pump was found to have no discernible toxic effects in tissues cultured on it *in vitro*.

15863

Mitotic Response to Colchicine in Human Cancer.*†

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Colchicine, the alkaloid isolated from the corm and seed of *Colchicum autumnale* L, has been known to arrest nuclear divisions in the metaphase stage in generative tissue of plants and animals. During the past 15 years the studies with colchicine initiated by Dustin, his collaborators and students have been amply tested, and frequently reviewed (Levine[1] and Ludford[2]). Here, only references pertinent to the problem under investigation will be made.

Colchicine and about 47 other chemical

* The aid rendered by the Social Service Bureau, Knights of Pythias, is gratefully acknowledged.

† We are indebted to S. B. Penick and Co. through the courtesy of Dr. George M. Hocking for some of the colchicine used in this work.

1 Levine, M., *Bot. Rev.*, 1945, **11**, 145.

2 Ludford, R. J., *J. Nat. Cancer Inst.*, 1945, **6**, 89.

substances recorded by Krythe and Wellensiek[3] influence nuclear divisions in a similar manner. Yet the usefulness of colchicine, in preference to the many chemicals with this singular property, has been pointed out by many workers and its application to the study of endocrinology, genetics, and cytology is well established. Its use in cancer therapy has been studied on tumors in animals and while it is generally conceded that these growths are arrested by this drug, the effect is only temporary. Repeated doses of colchicine seem to produce cumulative effects and have proved toxic.

Roentgenologists have contended that dividing nuclei are more vulnerable to the action of X-rays than are the resting nuclei in a given tissue. Since colchicine arrests the dividing nucleus in metaphase, and causes an accumulation of cells in this phase, the combination of colchicine and X-ray suggested itself as a feasible therapeutic measure. These agents have already been combined in the treatment of animal tumors (see Levine[4]) and in several cases of human cancer as reported by Brücke and Heuber[5] and by Seed, Slaughter and Limarzi.[6] The results in animals seemed to indicate that this procedure has some value. In normal plant tissues (Levine[7]) one of us has shown that the application of small quantities of colchicine combined with X-irradiation of 900r or 1500r arrests growth. Neither agent, alone, produces this effect.

It is now well known that the optimum influence of a given dose of colchicine as determined by the number of arrested mitoses varies with the species of animal or plant treated. In the mouse and rat the maximum effect is produced in 9 to 14 hours. The poikilothermal animals studied show the largest number of arrested dividing nuclei at 160 to 180 hours after the administration of a given dose of colchicine (Delcourt 1939). In the onion root tip, the maximum number of metaphases after colchicine occurs at the 24th hour (Levine and Gelber[8]).

Similar studies of the effect of colchicine on human cancer cells have been few and incomplete, so that the period at which the maximum number of metaphases results after a given dose of the drug is unknown. Oughterson, Tennant and Hirshfeld[9] studied colchicine effects on a group of 21 cancer patients: 15 had control biopsies taken before receiving colchicine and 11 of these showed arrested mitoses in metaphase. Colchicine was administered after the control biopsy; this was followed in 9½ hours by another biopsy or in some cases by the removal of the entire tumor. In one case, biopsies were taken before administration of colchicine and at 5, 9½ and 12 hours after an injection of 4 mg of the drug. Twenty oil immersion fields were studied from each biopsy and mitoses were counted. The control showed 2.6 mitoses per field, while 5 hours after the injection 7.3 mitoses per field were counted. Specimens taken at 9½ hours yielded 12 mitoses, while the 12-hour specimens post-colchicine, gave 19.6 metaphases. These workers state that colchicine injections make it possible in some instances to obtain a more accurate index of the rate of growth of the tumor. Seed, Slaughter and Limarzi,[6] it seems, used colchicine for its toxic effects on the tumor. They used the drug after X-rays were administered. Brücke and Hueber[5] colchicinized 2 comparable tumors and irradiated one. These authors contend that X-rays must follow colchicine and must be applied in the karyokinetic crisis. However, they took no such precautions in the irradiation of their patient. There was no apparent consideration given to the cytological status of the tumors treated. The question as to when X-ray therapy should begin has

3 Krythe, J. M., and Wellensiek, S. J., *Bibliog. Genet.*, 1942, **14**, 1.

4 Levine, Michael, *Cancer Res.*, 1945, **5**, 107.

5 Von Brücke, F. T., and Von Hueber, E. F., *Klin. Wchsch.*, 1939, **18**, 1160.

6 Seed, L., Slaughter, D. P., and Limarzi, L. R., *Surgery*, 1940, **7**, 696.

7 Levine, M., *Bull. Torrey Club*, 1945-1946, **72**, 563; **73**, 34; **73**, 167.

8 Levine, M., and Gelber, S., *Bull. Torrey Bot. Club*, 1943, **70**, 175.

9 Oughterson, A. W., Tennant, Robert, Jr., and Hirshfeld, John W., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 661.

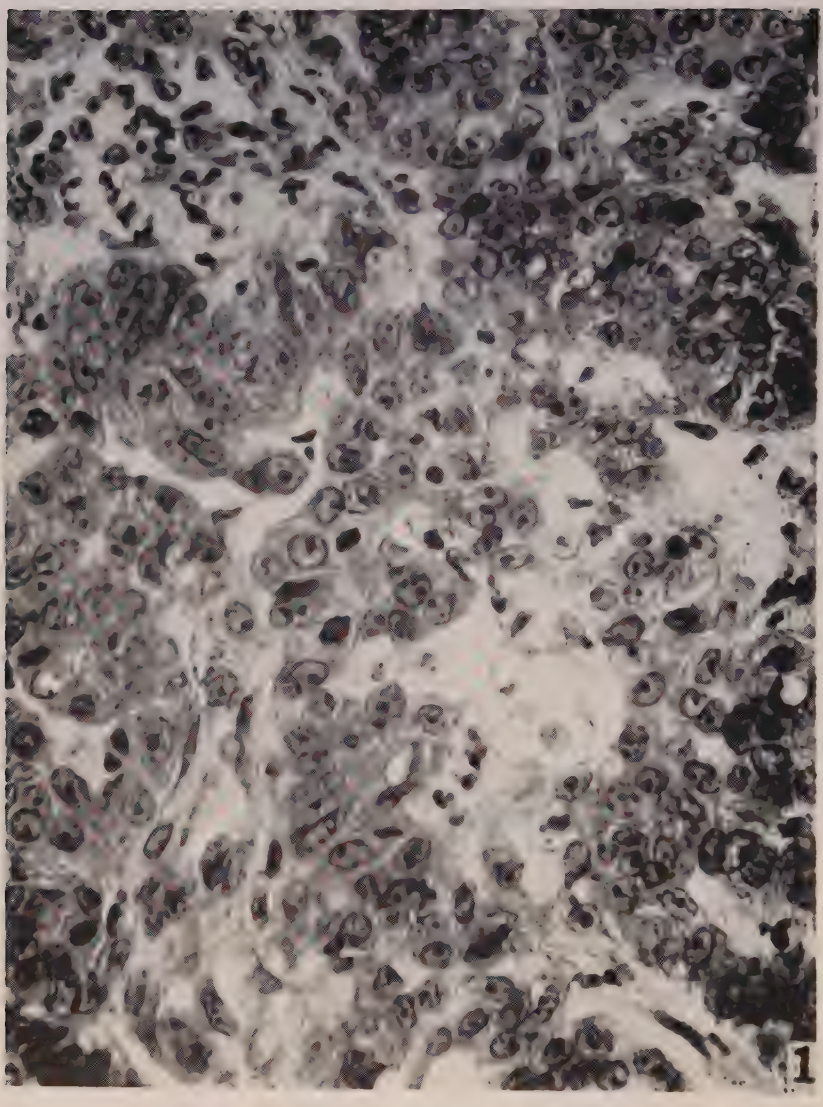

FIG. 1.
Case of B. G., photomicrograph of biopsy taken before the administration of colchicine. × 480.

not been considered. This appears significant in view of the contention that cells are most vulnerable to X-rays when the chromosomes are adequately exposed to these rays.

The following report deals with a study of the number of divisions (metaphases) in advanced carcinomas studied postoperatively following injections of 2 mg of colchicine. The primary purpose of this study is to determine as far as possible the time after colchicine administration when the greatest number of arrested mitoses appear in human cancer.

Methods and Materials. All the cases studied were postoperative, in terminal stages, with surface lesions. In preliminary studies on several of these patients mitotic counts were made at different times on different days without colchicine to verify the relative constancy of the number of division stages. No significant fluctuations were observed. After having made this observation, we studied the

TABLE I.
Average No. of Metaphases per H.P.F. Before and After 2 mg Colchicine.

Pt.	Diagnosis		Pre-colchicine	Post-colchicine in hr 4	8	12	16	20	24	32	48	72	96
BK	Ca bile duct		1.1	—	—	3.5	9.0	—	9.5	—	4.7	2.5	—
ML	Ca ovary		0.92	—	1.3	—	—	1.7	3.0	—	0.8	0.95	—
NT	Ca cervix		0.01	—	—	.01	2.2	0.8	0.1	—	0.1	0.6	—
JL	Epidermoid chin		0.0	0.4	0.8	1.0	0.8	1.7	1.2	—	1.4	0.9	—
CS	Ca rectum		0.72	0.9	1.7	1.1	1.2	—	3.0	—	1.0	1.2	0.45
RS	Ca sigmoid		1.3	5.5	5.2	13.4	—	14.3	13.8	5.9	1.3	1.4	—
YT	Epidermoid leg		1.1	1.2	2.0	1.6	2.1	2.2	2.2	1.8	3.9	4.7	2.3
				Partial Studies.									
BK	Ca bile duct	12/6/45											
			0.98				1.92						
		2/6/46											
			1.1				9.0						
BG	Ca sigmoid	3/12/46											
			6.1				21.4						
		3/19/46											
			5.6				39.0						

Pt.	Diagnosis	Pre-colchicine	Repeated doses of 2 mg colchicine at 24-hr intervals, followed by biopsy: 24 hr	48	72	96	120
MT	Ca cervix	0.43	2.1	2.0	3.2	3.7	1.6

response to 2 mg of colchicine introduced intramuscularly distant from the site of the neoplasm. Beginning at 4 hours after the colchicine injection, tissue was removed from the tumor at intervals of 4 hours for the first 24 hours and at daily intervals for the 2 days following. The tissues were fixed uniformly in Bouin's solution and imbedded in paraffin. Sections were cut 7½ μ in thickness and stained in Delafield's haematoxylin and eosin. A number of sections were mounted on each slide and the metaphases in 30 high power fields (H.P.F.) were recorded.

Observations. Of the 14 patients studied, 7 yielded suitable material for repeated counts, and the results are presented in Table I and composite graph. These were: one case of squamous cell carcinoma of the cervix, one adenocarcinoma of the rectum, one adenocarcinoma of the sigmoid, one carcinoma of the bile duct, 2 epidermoid carcinomas of the skin, and one adenocarcinoma of the ovary.

The metaphases counted in the precolchicinized tissue varied from zero to 1.3 per H.P.F. The first 10 hours after the treatment showed a slight increase in the mitotic count. Beyond this time the number of metaphases increased until a maximum was reached between the 16th and 24th hours. After that, there was a decline in the number of nuclei in this phase. At 72 hours after a single dose of colchicine, the count was still slightly greater than that at the beginning of the experiment. There was in general a uniform response to the colchicine as indicated in Table I with the maximum occurring most often at 24 hours after the injection. The number of cells that responded, however, varied considerably with the patient or the tumor and ranged from 1.7 to 14.3 per H.P.F. at this period. While the karyokinetic activity varied in number, 5 cases were proportionately similar. In 2 cases of the 7, B.K. and R.S., the number of metaphases was 10 times greater than in any of the other patients studied. The metaphase count in the case of Y.T. differed from all the other patients: starting from a value of 1.1 metaphases in the control biopsy, a first peak of 2.1 was reached at 16 hours, and a second one of 4.7 in 72 hours. The specimen obtained 96 hours after colchicine administration was still elevated and gave a count of 2.3 metaphases.

In the case of B.K. indicated in Table I

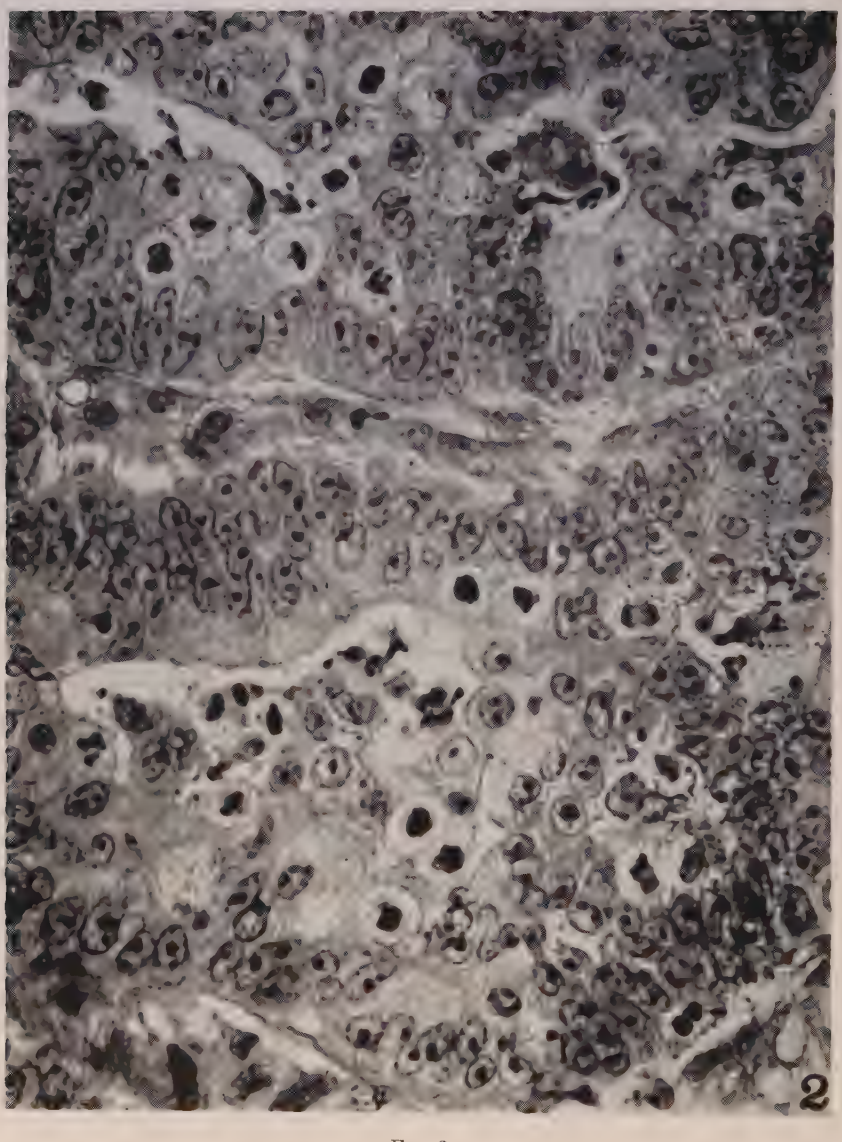

FIG. 2.
Same case, biopsy taken 7 days later, 16 hours after colchicine. × 480.

under "partial studies" 2 biopsies taken a day apart before administration of colchicine gave an average of 0.78 and 0.98 metaphases. Sixteen hours after colchicine the metaphase average increased to 1.92 per H.P.F. Two months later this patient was tested again: the control count revealed 1.1 divisions but at 16 hours after another injection of colchicine, the count rose to 9.0. Similarly in another case, B.G., the counts before and after 2 separate colchicine injections seemed significant. In this case the control biopsy made on March 12 gave an average of 6.1 metaphases (Fig. 1). After the injection of colchicine a second biopsy 16 hours later gave an average of 21.4 metaphases per H.P.F. Following a rest of 7 days, another biopsy from this case showed an average of 5.6 metaphases. This was followed by a second injection of a similar quantity of colchicine,

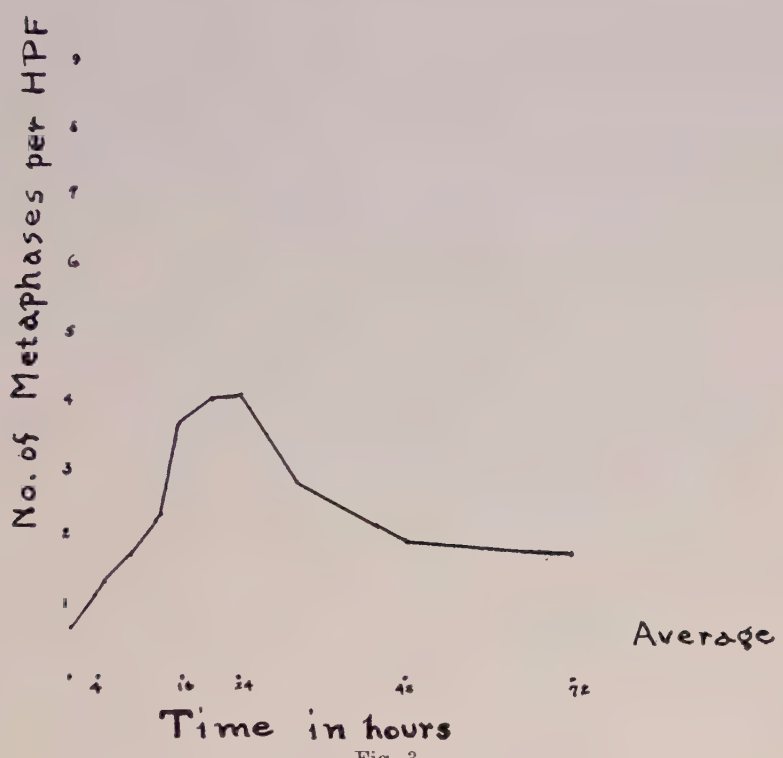

Fig. 3.

and 16 hours later another biopsy was done. The tissue showed an average of 39.0 metaphases per H.P.F. (Fig. 2).

It is of interest to note in both cases, B.K. and B.G., the second dose of colchicine gave a high metaphase count as compared with that found after an initial injection of the drug. The tumor tissue studied after a colchicine injection showed considerable hemorrhage and invasion of the tumor tissue by numerous white blood cells.

M.T., a case of cancer of the cervix with spread to the vulva, was biopsied at the site of the secondary tumor. The control section had very few division figures: no metaphases in 30 H.P.F. studied. The biopsy 12 hours after the injection of colchicine showed no change. The 16-hour biopsy had an average of 2.2 metaphases. Biopsies which followed at the 23rd, 44th and 69th hour showed no increase in the metaphase number. The small number of metaphases per field after colchicine seemed to indicate an exceedingly slow growing tumor and it appeared of interest to determine the influence on this tumor of repeated doses of this drug. After a rest of 30 days with the condition of the patient and the macroscopic appearance of the tumor unchanged, a biopsy was taken. This was followed by an injection of 2 mg of colchicine on 6 successive days. The results of these observations are likewise listed in Table I under "partial studies." The number of metaphases per H.P.F. prior to injection of colchicine was 0.43. The succeeding biopsy showed a rise to 2.1 metaphases per H.P.F. and after the 4th injection 3.7 metaphases were counted per H.P.F. This was followed by a decline to 1.6, 24 hours after the 5th injection. The microscopic preparations of this tissue contained extensive hemorrhage. The cancer tissue showed late mitotic stages.

Summary and Conclusions. 1. These observations seem to indicate that a single intramuscular injection of 2 mg of colchicine induces an arrest of nuclear division in the metaphase stage of human cancer. The number of cells in this stage increases slowly and the effect reaches a maximum between the

16th and 24th hours. The decline in number of metaphases is more gradual than the rise.

2. Repeated injections of colchicine in some patients after a relatively short rest period indicate a possible increased sensitivity to the drug.

3. Biopsies taken 48 to 72 hours after colchicine show an increase in hemorrhage, leucocytosis and some polyploid cells. Late telophases are occasionally observed in this colchicinized tissue.

4. The evidence presented here suggests the basis for further study of the effect of colchicine combined with other physical or chemical agents in the treatment of human cancer.

15864

Tantalum Oxide and Wound Healing: Experimental Study.

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Tantalum is a metallic element having an atomic weight of 180.88 and a density of 16.6. It has had some use in surgery, chiefly in the form of plates for substitution in cranial defects. It has been praised as being biologically inert.

Olson[1] reported that tantalum foil placed over wounds from industrial accidents where there had been partial loss of thickness of the skin seemed to produce epithelialization faster than any other method of treatment. It was assumed that this apparent stimulating action was due to the oxide coating of the tantalum which is always present. He then prepared a fine tantalum oxide powder and used it in treating small wounds and burns of the extremities. He reported that the acceleration of healing and the absence of pain were noteworthy. Since his studies did not include controls, we decided to do the following wound healing experiments.

Technic. The technic described by Brush and Lam[2] was used to test the effect of tantalum oxide powder on wound healing in 47 guinea pigs.

Under ether anesthesia the abdomen was shaved and treated with an antiseptic (hexylchloro-m-cresol). Using sterile scissors, an oval wound measuring approximately 8 x 10 mm in diameter and going through to subcutaneous tissue was made on each side of the midline. Fig. 1 illustrates the appearance of the wounds immediately after being made.

According to Carrel's law it is unnecessary for the wounds to be exactly similar because wounds in the same individual, even if of different size, tend to heal in the same period of time. The wound on the left of the animal was used to test the tantalum oxide, the right wound being the control. The outline of each wound was traced on sterile cellophane; then the tantalum oxide powder was placed on the left wound, and sterile gauze

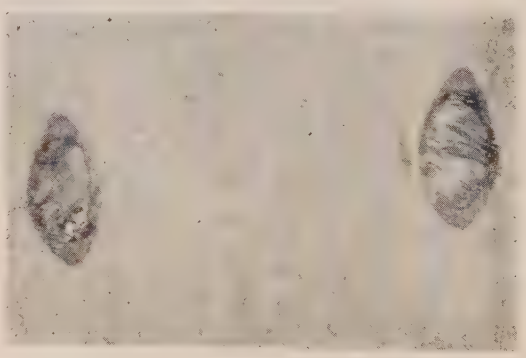

FIG. 1.
The appearance of the wounds just after being made.

[1] Olson, C. T., *Industrial Medicine*, 1945, **14**, 949.

[2] Brush, Brock E., and Lam, Conrad R., *Surgery*, 1942, **12**, 355.

TABLE I.
Healing of Control Wounds Versus Tantalum Oxide Treated Wounds in Series I (12 animals). (Dressings changed every 2 days).

Both wounds healed on same day	8 animals
Tantalum oxide treated wounds healed first (one dressing period ahead of control wound)	4 animals
	12

TABLE II.
Days Required for Wound Healing in Series II (12 animals).
(Wounds dressed on 8th, 12th, 17th, 22nd, 25th and 27th day.)

Healing	Control wound (days)	Tantalum oxide treated wound (days)
Hastened	25	17
	27	17
	22	17
Unchanged	14	14
	12	12
	12	12
	12	12
	17	17
	12	12
	12	12
Delayed	12	14
	12	14

on the right wound. The tantalum oxide powder was sterilized by heating to 400°F for one hour in a dry oven. The wounds were redressed and measured by tracing every two days. Fresh tantalum powder was applied after each tracing. Table I demonstrates the results in the first series of 12 guinea pigs.

It was then suggested that perhaps the frequent dressing (every 2 days) interfered with the healing rate by pulling off granulation tissue and exposing to infection. We therefore waited 8 to 11 days before changing the first dressing on the remainder of the animals tested. Table II represents a typical experiment on 12 animals (Series 2) showing the number of days required for the complete healing of each wound.

TABLE III.
Comparing the Rate of Healing of Tantalum Oxide Treated Wounds with Control and with Talc Treated Wounds (Series III—23 animals).
(Wounds dressed on 7th, 11th, 14th, and 17th days.)

All wounds healed on same day	10 animals
Tantalum oxide wound healed first	5
Talc treated wound healed first	2
Control wound healed first	1
Tantalum oxide wound healed last	3
Talc treated wound healed last	1
Control wound healed last	1
	23

To determine if the supposed beneficial action of tantalum oxide powder was due only to the drying action of any fine powder, we then modified the experiment to include another wound on the animal's abdomen which was treated with talc. The talc was sterilized in the same manner by heating to 400°F for one hour. Three wounds of equal size were made on the guinea pig's abdomen, avoiding the less vascular midline. The central wound was used as a control, tantalum oxide powder placed in the left wound and talc in the right wound. Again, the first dressing was delayed until the 10th day. Table III sums up the result of healing of the wounds treated in this manner.

In the total number of 47 guinea pigs tested, the tantalum oxide-treated wounds healed first in 12 pigs, the control or talc-treated wounds healed first in 8 pigs, and in the 27 remaining animals there was no appreciable change in rate of healing.

Summary. Small cutaneous defects on the abdomen of guinea pigs were treated with sterile tantalum oxide powder. The healing time of these wounds was compared with that of control wounds dressed with sterile gauze or sterile talc. No consistent acceleration of healing of the tantalum oxide-treated wounds could be demonstrated under the conditions of the experiment.

15865 P

Effect of Urethane on Maturation of Leukocytes of Mouse Myelogenous Leukemia.*

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Urethane induces a pronounced depression in the blood leukocyte count of both human and mouse myelogenous leukemia.[1,2] In the experiments being reported, mice with myeloid leukemia were given either 1 or 2 anesthetic doses of urethane (1 mg per g of body weight intraperitoneally in 10% aqueous solution) and the alterations in the white blood cells of bone marrow and peripheral blood observed. The leukemic animals were of the second transfer generation of a myeloid leukemia of the F strain.[3] The F mice of these experiments had been inoculated with leukemic cells to effect transfer of the disease 34 to 44 days before they were placed on experiment; animals were from 6 to 8 weeks of age at the time of inoculation. They were weighed daily after treatment with urethane was begun.

Total white blood cell counts were made on the tail blood of 9 leukemic mice preceding and at 24-hour intervals (from 24 to 192 hours) after a single anesthetic dose of urethane. Differential and total blood leukocyte counts were made on nonleukemic animals preceding and 24 hours following 1, 2, 4 or 5 daily anesthetic doses. Differential counts were made on the bone marrows of 11 untreated leukemic mice, and of 12 leukemic mice within 11 to 31 hours after either a single or 2 anesthetic doses of urethane spaced at 24-hour intervals. Thin films of sternal marrow were smeared upon glass slides and stained with May-Grünwald Giemsa staining combination. In doing a differential count the myeloid cells with a single round nucleus (blast cells) were counted against those with a nucleus which had segmented. The number of mitotic figures in 40 oil immersion fields was determined on the marrow of these animals. Similar determinations of mitotic figures were made on the bone marrow of 6 nonleukemic mice which had received no treatment and 6 which had received one injection of urethane 11 hours previously.

A single anesthetic dose of urethane depressed the white counts of leukemic mice from levels of 78 to 286,000 to 23 to 44,000 within 72 hours (uniform response in 11 mice with an average drop of 106,000 in the first 24 hours). Most of the "blast" cells disappeared from the circulating blood. There was a loss of about 1 g in weight during this period (initial weight of 21 g). During the next 72 hours the counts rose, although in 6 of the 9 mice studied in this manner the counts had not reached the initial levels by this time.

The ratio of segmented to mononuclear cells in the marrow of untreated leukemic mice ranged from 13:87 to 46:54 with an average of 26:74. This ratio was altered (37:53 to 76:24 with an average of 60:40), indicative of a shift towards maturity, within 11 to 48 hours after either a single or 2 injections of urethane spaced at 24-hour intervals. These doses did not alter the white blood cell counts of normal mice, although 4 or 5 daily injections induced a depression in the white blood cell counts of nonleukemic animals. When treatment of leukemic mice with urethane was suspended (192 hours after a single anesthetic dose), mononuclear myeloid cells again pre-

* This investigation has been aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research, the National Cancer Institute, and the Cancer Fund of the Graduate School of the University of Minnesota.

† Fellow of the China Medical Board.

1 Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **250**, 677.

2 Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

3 Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.

dominated in the bone marrow.

Urethane has been observed to inhibit mitotic activity in the corneal epithelium of the rat,[4] and similar effects have been noted for other cells in tissue culture.[5] Consequently counts of mitotic figures were made on the bone marrow of normal and leukemic mice before and after administration of urethane. No inhibition of mitosis in myeloid cells could be detected 11 hours after a single injection of urethane into normal mice (6 animals). Fewer mitotic figures were present, however, in the marrows of urethane-treated leukemic mice (11-24 hours after last injection) than in controls (29 mitotic figures for 40 oil immersion fields of untreated leukemic marrows—range of 12 to 42, 14 mitoses in treated marrows—range of 7 to 30). This is not interpreted necessarily as meaning that mitosis had been inhibited by the drug, since there were fewer cells in the urethane-treated marrows capable of undergoing mitotic division.

As a result of the action of urethane many mature cells appeared in the bone marrow. With the release of these cells instead of immature leukemic cells into the blood, the circulating population of white blood cells had a shorter life expectancy. Thus, a great per cent of the circulating cells were probably dying within a relatively short time after release from the marrow. This is not offered as the probable sole explanation for the depression in the white blood cell count of myeloid leukemia following urethane therapy, since other factors such as rate of release of cells from the marrow and peripheral destruction may be involved.

These animals with myeloid leukemia represent an excellent test object for determining the ability of drugs chemically related to urethane to produce similar effects on myeloid leukemia.

Summary. The administration of a single anesthetic dose of urethane resulted within 24 hours in a drop in the white blood cell count and the appearance of many mature cells in the bone marrow of leukemic mice. Since the number of mitotic figures in marrow myeloid cells was decreased, maturation may have been secondary to inhibition of mitosis in blast cells. However, in the treated mice there were fewer marrow cells capable of undergoing division, which may account for the reduced number of mitoses. The release of an increased per cent of mature cells into the circulating blood may be a factor in depression of the white blood cell counts following the injection of urethane into mice with myeloid leukemia.

4 Guyer, M. F., and Claus, P. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 3.

5 Ludford, R. J., *Arch. f. exp. Zellforsch.*, 1936, **18**, 411.

15866 P

The Oxigram as a Measure of Cardiorespiratory Reserve.*

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Most of the available methods for determining the functional reserve of the cardiorespiratory system have objectionable features.[1-6] A new test was suggested by the consideration that in disease of the heart or

* Aided by a grant from the Dazian Foundation for Medical Research.

1 Nylin, G., *J. A. M. A.*, 1937, **109**, 1333.

2 Schneider, E. C., *J. A. M. A.*, 1920, **74**, 1507.

3 Master, A. M., *Am. Heart J.*, 1935, **10**, 495.

4 Master, A. M., Friedman, R., and Dack, S., *Am. Heart J.*, 1942, **24**, 777.

5 Levy, R. L., Bruenn, H. G., and Russell, N. G., Jr., *Am. J. Med. Sc.*, 1939, **197**, 241.

TABLE I.

Analysis of Oxigrams of 6 Normal Subjects and of 3 Patients. The normal values in the cardiac patient (V.P.) with good estimated reserve (class IB) are to be noted.

Subject	Diagnosis*	Lowest O_2 saturation during 5 min. hypoxia	"O_2 Unsaturation Min." during 5 min. hypoxia
S.B., ♂, 23 yrs	Normal	82.5	62.6
C.E.K., ♂, 37 "	"	87.0	34.0
J.B., ♀, 38 "	"	89.5	38.5
B.G. ♂, 21 "	"	81.0	70.0
H.N., ♀, 22 "	"	91.5	29.0
A.H., ♂, 25 "	"	84.0	56.0
		Mean 85.9	Mean 48.4
V.P., ♂, 23 "	Inactive rheumatic heart disease, class IB	88.0	39.4
W.O'B., ♂, 65 "	1. Hypertensive and arteriosclerotic heart disease, class IVD 2. Pulmonary emphysema	65.0	142.8
L.B., ♂, 81 "	1. Arteriosclerotic and hypertensive heart disease, class IVE 2. RLL pneumonia	75.0	91.0

* Functional and therapeutic diagnoses in accordance with "Nomenclature and Criteria for Diagnosis of Diseases of the Heart."

of the lungs the form of hypoxia encountered is of the hypoxic or stagnant type, or a combination. In either, the extent of the abnormality is reflected in the degree of hypoxemia. A continuous record of the oxygen saturation of the arterial blood in response to the inhalation of gaseous mixtures with different tensions of oxygen might give a quantitative measure of the efficiency of oxygen transport, and in turn of cardiac and of respiratory function and functional reserve. A record of the kind in question we have termed the "oxigram."

An oxigram is easily made with the aid of Millikan's oximeter.[7] The method is quite accurate, for simultaneous determinations on normal subjects[8] at various levels of oxygen saturation down to 60% by means of the oximeter and of the gasometric method[9] have revealed differences usually within ±5%.

Adaptation to a Clinical Test. Low oxygen tension mixtures as a means of testing the efficiency of the hemal oxygenating mechanisms were administered. The procedure was as follows: The galvanometer having been adjusted, the photometer was placed on the ear of the sitting subject or patient. For 15 or 20 minutes, 100% oxygen was breathed through a BLB mask (Army Air Forces type A-8B)† and when no further rise of the galvanometric deflection occurred the subject's arterial blood was assumed to be saturated 100% of capacity. The mask was removed and room air breathed until the galvanometer levelled off. The mask was then replaced and connected to a source of gas containing 10% oxygen (pO_2 approximately 76 mm Hg) and 90% nitrogen (pN_2 approximately 684 mm Hg). In normal subjects this mixture was breathed as long as possible, but in patients the degree of hypoxemia induced could not be tolerated for more than 5 minutes. It is probable, therefore, that as finally evolved the test period

6 Caughey, J. L., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 973.

7 Millikan, G. A., *Rev. Sc. Instruments*, 1942, **13**, 434.

8 Hemingway, A., and Taylor, C. B., *J. Lab. and Clin. Med.*, 1944, **29**, 987.

9 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **64**, 543.

† This mask probably does not deliver the exact percentage of gas found in the oxygen bottle attached to it. See reference.[10]

10 Army Air Forces Manual No. 25-0-1, Nov., 1945, and Manual No. 25-2, 15 March, 1945, Headquarters, Army Air Forces, Washington, D.C.

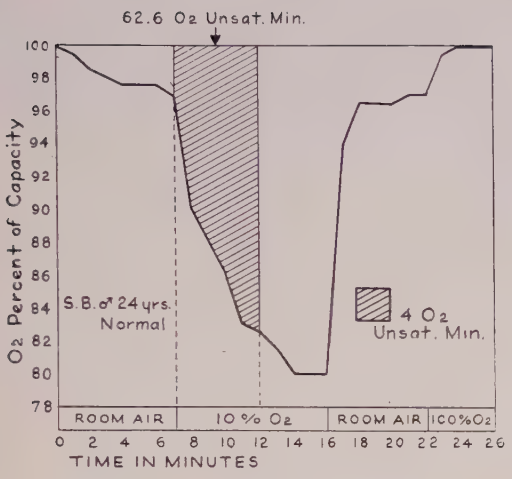

FIG. 1.
Oxigram of a young normal subject.

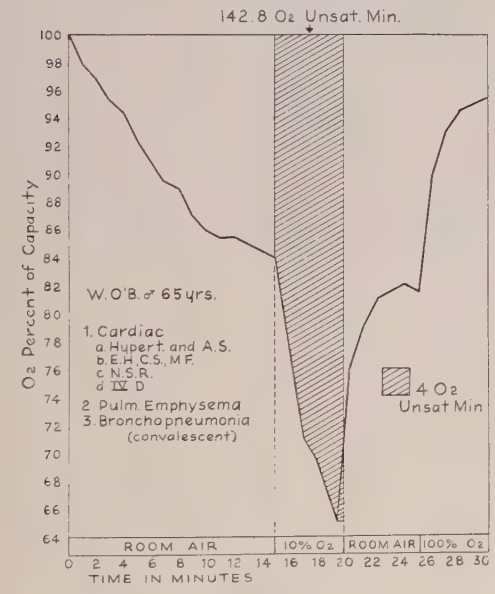

FIG. 2.
Oxigram of a cardiac patient convalescing from bronchopneumonia. The oxygen-deficient mixture could not be breathed for more than 4.5 minutes. The 5-minute area was completed by merely extending the lowest oxygen saturation reached to the end of the fifth minute.

will be limited to 5 minutes in duration since even in normal subjects, the steepest part of the unsaturation curve is encountered in this time. Following the test period, room air was breathed again until pretest levels were reached. Finally 100% oxygen was given by mask for several minutes, largely as a check on the accuracy of the apparatus.

Results. Representative oxigrams are shown in Fig. 1 and 2 and the results obtained in 6 normal subjects and in 3 patients are shown in Table I. The differences between the normal (Fig. 1) and the cardiac with good reserve (Table I) on the one hand, and the patient with poor cardiac reserve on the other (Fig. 2) are immediately apparent. Particularly notable in the latter are the considerable fall of the curve while the patient was breathing room air; the depth to which the curve descended during the actual test period; and the sluggish return to pretest levels when the patient was permitted to breathe room air again.

In order to get a measure of the rate as well as depth of fall of the oxigram in response to induced hypoxemia various areas of the curve in units of "oxygen unsaturation minutes" were measured with a planimeter. Taking the immediate prehypoxic level of oxygen saturation as a base it was found that the area of the curve during 5 minutes of hypoxia did not differ greatly in normal subjects and in patients with cardiopulmonary disease. Clearly, however, a sufficient number of cases have not been tested. In order to include into a single measurement the oxygen unsaturation that occurred when the subjects were breathing room air and when they were breathing low oxygen mixtures, the area of the test curve was extended to the baseline of 100% saturation (see shaded areas in figures). There was then, of course, considerable difference between patients with poor reserve and normal subjects.

Establishment of the reliability of the procedure, and correlation with other measures of cardiac and respiratory function remain to be done.

Summary. A graph in which oxygen saturation of the arterial blood is plotted against time while the subject is breathing different concentrations of oxygen in a standard sequence is termed an "oxigram." Preliminary observations indicate that the oxigram may be useful for measuring cardiorespiratory reserve.

The authors are grateful to Doctor Glenn A. Millikan of Vanderbilt University for permission to use some of his apparatus.

15867 P

Quantitative Studies of Complement Fixation.*

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The spectrophotometric method for the precise estimation of the hemolytic activity of complement[1,2] has now been applied to a quantitative study of complement fixation by the interaction of single purified antigens and homologous and cross-reacting antibodies.

As in the classical complement fixation technic, a constant amount of complement (C′) is employed. However, sufficient C′ (usually 50 50% hemolytic units) is added to avoid complete fixation. The extent of fixation is determined by quantitative estimation of the residual hemolytic activity and subtraction of this value from the mean value of the C′ activity buffer, antigen and antibody controls. The results, expressed as the number of C′ units fixed, are more readily interpretable than the indirect expressions employed by previous investigators.[3,4] This method, moreover, permits the use of a single table of conversion factors as calculated from von Krogh's equation with a value for $1/n = 0.2$.[2]

Materials and Methods. Standardized suspensions of sheep erythrocytes are prepared as in [1]. Since Ca^{++} and Mg^{++} are essential constituents of the hemolytic system,[2] these ions are supplied in optimal concentrations by the use of veronal buffer[2] containing 0.00015 *M* $CaCl_2$ and 0.0005 *M* $MgCl_2$ as diluent for all reagents used in the test.

2.5 ml† of a dilution of immune rabbit serum (in this instance antipneumococcus Type III) are mixed in the cold in 25 x 115 mm tubes with 5.0 ml of a dilution of guinea pig C′ containing 50 50% hemolytic units; 2.5 ml of S III‡ solution are then added with thorough mixing. The tubes are incubated at 37 ± 0.1°C. After 60 minutes§ the tubes are chilled in ice-water. A measured volume of the contents of each tube is diluted with chilled isotonic buffer so that aliquot portions of this dilution will cause partial lysis in the range of 20-80% and thus furnish analyses appropriate for the estimation of the 50% hemolytic unit of the residual C′. The hemolytic reaction is conducted as in [2] except that the total volume is 7.5 ml instead of 5.0 ml.

Details of procedure and calculations of a typical experiment are illustrated in Table I.

Results. 1. With a constant quantity of S III, the units of C′ fixed increase as the amount of antibody N is increased. With 0.25 μg S III, the increase in fixation is linear in the range of 0.5 to 1.5 μg of antibody N. The linear relationship might be utilized for measurements of minute amounts of antibody.

2. No fixation of C′ occurs when the ratio of antibody N to S III approaches 2 or less, in the region of extreme antigen excess.

Data have also been obtained with constant amounts of antibody and varying quantities of S III, with varying numbers of

* Carried out in part with the aid of a grant from the Rockefeller Foundation and in part under the Harkness Research Fund of the Presbyterian Hospital, New York City.

1 Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

2 Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

3 Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1938, **35**, 217, and other papers.

4 Rice, C. E., *J. Immunol.*, 1947, **55**, 1, and earlier papers.

† All measurements are made with calibrated pipettes and all glassware is cleaned with sulfuric acid-sodium dichromate cleaning mixture.

‡ SIII = specific polysaccharide of Type III pneumococcus.

§ In subsequent studies a fixation period of 90 minutes was used.

TABLE I.
Units of Guinea Pig C′ Fixed by 0.25 μg S III and Varying Quantities of Rabbit Antipneumococcus Type III Serum C-28.

Controls		Titration residual C′: Dilution of reaction mixture	Vol. of dilution tested, ml	Veronal buffer added, ml		Hemolysis %	Factor†	Residual C′, units	C′ fixed, units	C′ fixed corr. to 50 units, units
Serum (4 μg antibody N)		1 + 9	2.0	4.5	1.0 ml hemolytic system added. Incubation 60 min.* at 37°C.	45.9	0.970	48.5		
			2.5	4.0		71.8	1.202	48.1		
S III (0.25 μg)		1 + 9	2.0	4.5		49.5	0.998	49.9		
			2.5	4.0		72.8	1.215	48.6		
Buffer		1 + 9	2.0	4.5		47.1	0.978	48.9		
			2.5	4.0		71.8	1.202	48.1		
							Mean:	48.7		
Antibody N used, μg	Fixation 60 min. at 37°C.									
0.71		1 + 9	2.0	4.5		39.5	0.915	45.8	3.5	3.6
			2.5	4.0		63.3	1.113	44.5		
0.94		1 + 9	2.5	4.0		52.4	1.02	40.8	7.8	8.0
			3.0	3.5		73.9	1.23	41.0		
1.18		1 + 9	3.0	3.5		60.7	1.090	36.3	12.8	13.1
			3.5	3.0		74.9	1.241	35.5		
1.66		1 + 9	3.5	3.0		43.5	0.950	27.1	21.9	22.5
			4.0	2.5		56.9	1.056	26.4		
2.36		1 + 4	3.0	3.5		59.5	1.078	18.0	30.7	31.5
			3.5	3.0		76.0	1.260	18.0		
3.78		1 + 4	3.5	3.0		47.5	0.982	14.0	34.7	35.6
			4.0	2.5		64.4	1.125	14.0		

* In other experiments 45 min. were shown to be adequate.
† From Reference 2, based on $1/n = 0.2$.

C′ units, and with other immune systems, and these are being assembled for publication.

Summary. A quantitative method for the determination of complement fixation is briefly described and data are given for its application to one immune system.

15868 P

Histochemical Demonstration of Liver Glycogen in Human Diabetic Acidosis by Liver Biopsy.

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In spite of the extensive experimental and clinical literature which has been devoted to diabetes mellitus, few observations have been concerned with liver glycogen in human diabetes. These were based on autopsy material or on single specimens removed at operation. Two recently developed technics make the direct study of the liver glycogen content in the human feasible under clinical conditions. The 3-inch Silverman biopsy needle[1] makes it possible to obtain enough tissue for histologic examination; and Gomori's histochemical method for the demonstration of glycogen[2] appears to permit a quite accurate estimate of the glycogen content.

Method. A core of liver tissue is secured by the Silverman needle, at varying times during the treatment of patients in diabetic acidosis. Although needle biopsy is a reasonably safe procedure, it should be performed cautiously, and only after defects of the clotting mechanism have been ruled out. Moreover, the risk may be greater in unconscious patients.

The tissue, fixed for 24 hours in absolute alcohol, is stained by Gomori's method. Blood sugar levels are determined by the method of Folin and Wu,[3] and plasma CO_2 combining powers by the manometric Van Slyke technic.[4]

Results. Liver biopsies have been performed in diabetic patients with acidosis, and the histologic results correlated with the clinical and biochemical status of the patient. An example of the data so obtained is shown in Fig. 1. This patient was a 35-year-old Negro woman, not previously known to be diabetic, who had had polyuria and polydipsia for 4 months. During the 10 hours prior to admission she had been comatose. No evidence of infection was found. Fig. 1-A shows the liver glycogen prior to treatment. At this time, the blood sugar level was 358 mg/100 cc, the CO_2 combining power 27.8 vol % (11.6 mM/L) and there was 4+ glycosuria and 2+ acetonuria. Note that although the glycogen content is greatly diminished, a small amount is still present. Many large vacuoles, indicating fatty metamorphosis, are seen in the liver cells of the midzonal portions of the lobules.

Fig. 1-B shows the appearance of the liver 7½ hours later. By this time, the patient had received 300 units of regular insulin, 4000 ml saline and 300 g of glucose by vein. Her urine had been acetone-free for 2½ hours, but still showed 4+ test for sugar. The CO_2 combining power was 43.2 vol % (19.3 mM/L). Note the striking restoration of glycogen. Fat vacuoles are still numerous. A third biopsy (Fig. 1-C) was taken after 6 days, when the diabetes had been well controlled for 3 days. Note that there

[1] Silverman, I., *Am. J. Surg.*, 1938, **40**, 671.

[2] Gomori, G., *Am. J. Clin. Path.* (Tech. Sect.), 1946, **10**, 177.

[3] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

[4] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

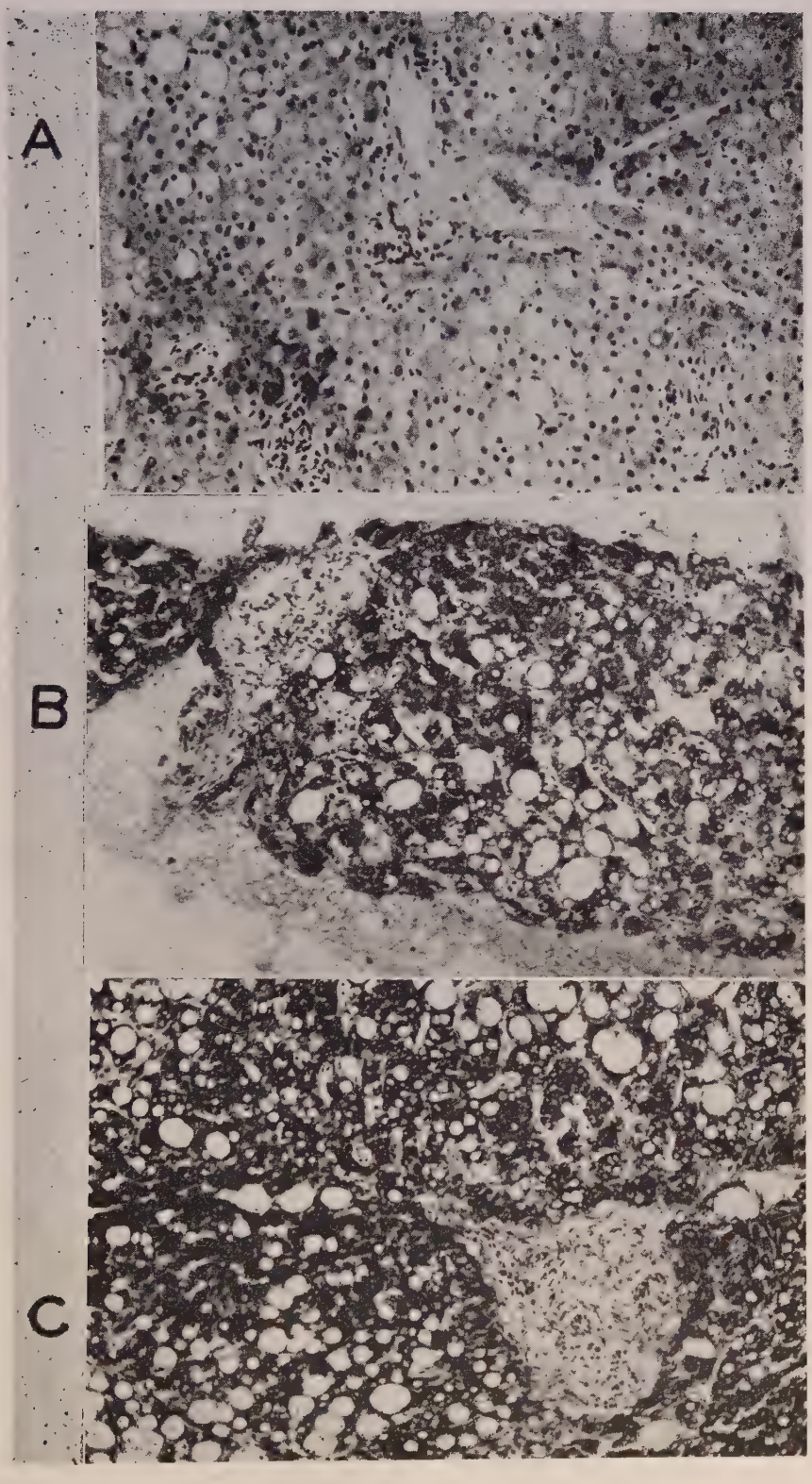

FIG. 1.

A. Glycogen depletion and fatty metamorphosis in liver during acidosis. B. Restoration of glycogen with persistent fatty metamorphosis after 7½ hours of treatment. C. Slight further increase in glycogen 6 days later. The black granules in the cytoplasm represent glycogen. Gomori's glycogen test, × 160.

is only a slight increase in glycogen content and no change in the degree of fatty metamorphosis.

Discussion. In order to evaluate these findings, knowledge of normal values and their fluctuations is essential. The only observations of the glycogen content of the liver in human beings have been obtained at operation, when the metabolism had been deranged by operative trauma, anesthesia, and by previous illness.[5] Autopsy material may not give an accurate picture because of the rapidity of postmortem glycogenolysis, and because even brief agonal states are known to cause drastic changes in the liver glycogen of animals. We are, therefore, accumulating a series of observations in normal individuals in the postabsorptive state, after meals, and after the administration of various drugs and stresses.

A quantitative correlation between histochemical and chemical methods for the determination of liver glycogen would be desirable. The study by Deane, Nesbett and Hastings[6] indicates that a good correlation can be obtained. To this end, we are performing simultaneous quantitative and histochemical determinations of glycogen in livers obtained from rats under various experimental conditions, as well as from human tissue obtained at autopsy. To date, it appears that a roughly quantitative correlation can be observed, and it is hoped that ultimately this can be done with a fair degree of accuracy.

Summary. Liver biopsy specimens obtained during diabetic acidosis and its treatment were examined by the Gomori technic for glycogen. Severe glycogen depletion was found before treatment. Restoration of glycogen content occurred after a few hours of therapy.

[5] MacIntyre, D. S., Pedersen, S., and Maddock, W. G., *Surgery*, 1941, **10**, 716.

[6] Deane, H. W., Nesbett, F. B., and Hastings, A. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 401.

15869

Scleroma: Complement Fixation Test.

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From the time that rhinoscleroma was discovered in 1870 by von Hebra,[1] the disease has been the subject of a good deal of controversy. The name of the disease itself has been considered inadequate, and in 1932, at the Second International Congress Otorhinolaryngology[2] held in Madrid, it was changed to the more inclusive term, scleroma. The lack of definite information on the etiology of the disease has probably been the main source of confusion. Despite the inclusion of the supposed causative organism, *Klebsiella rhinoscleromatis*, in Bergey's Manual,[3] this bacillus is far from acceptable as the etiologic agent. Wilson and Miles[4] say, ". . . there is very little evidence that this organism is primarily responsible for it. There is no means by which it can be distinguished with certainty from other members of the capsulated group; and, since we know that members of this group may be present in the nose of healthy persons, it is difficult to prove

[1] von Hebra, R., *Wien. med. Wchnschr.*, 1870, **20**, 1.

[2] Rapp, Cong, *Madrid Internat. d'Oto-rhenolaryng.*, 1932.

[3] Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, Baltimore, 1939.

[4] Wilson, G. S., and Miles, A. A., *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd Ed., Baltimore, 1946.

TABLE I.
Complement Fixation of Routine Patients' Sera.

No. of sera	Serum titration 2.5	5	10	20	40	80	160	320	640
5	1+	1+							
3	2+	1+							
1	2+	2+							
1	3+	3+	3+	3+	2+	0			
1	3+	3+	3+	2+	0				
1	3+	3+	0	0					
1	4+	4+							
*1	4+	3+							
*1	4+	4+							

* On repeating with new samples of sera, titration was negative.

TABLE II.
Complement Fixation of Sera from Patients with Scleroma.

No. of case	Serum titration 2.5	5	10	20	40	80	160	320	640	1280
1L	4+	4+	4+	4+	4+	2+	1+	0		
2M	4+	4+	4+	4+	4+	4+	2+	0		
3F	4+	4+	4+	4+	4+	4+	1+	0		
4J	3+	2+	1+	1+	0	0	0	0		
5C	4+	4+	4+	4+	4+	1+	0	0		
*6M	4+	4+	4+	4+	2+	1+	0	0		
7	4+	4+	4+	4+	4+	3+	3+	0		
8	4+	4+	4+	4+	4+	4+	4+	3+		
9	4+	4+	3+	3+	1+	0	0	0		
10	4+	4+	4+	4+	4+	4+	4+	4+	2+	1+
†11	4+	4+	4+	4+	4+	4+	4+	4+		

* Cases 6M, 7, 8, and 9 submitted by Dr. M. Ruiz Castaneda of Mexico City.
† Case 11 submitted by Dr. M. Gerundo, Hilo, Hawaii.

that they play any part in production of rhinoscleromatis."

We have studied many phases of the problem and have reported our conclusions in a publication now in press.[5] We have described an organism (isolated from cases of scleroma) which we feel to be the cause of the disease. It is a Gram-negative, nonmotile rod, forming mucoid colonies on nutrient agar and eosin-methylene-blue agar. It ferments maltose, mannite and dextrose, but never lactose. Occasionally sucrose is fermented. All sugars fermented form acid only.

This paper is intended to describe a complement fixation test for scleroma in which the above bacterium is used as the antigen.

Experimental Procedure. The organism used was isolated from a patient with scleroma. It was grown in nutrient broth containing 5% dextrose for 7 days. At the end of that time it was centrifuged clear of the supernatant which was discarded. The organism was then washed twice in saline, each washing equivalent in amount to the original broth. The organism was exposed to 65°C for 30 minutes and suspended in 1:5000 merthiolate in .85% saline. Organisms from 10 liters of the original broth were resuspended in 100 cc of the merthiolate-saline solution. An anticomplimentary titer was now run. The antigen suspension was found to be anticomplementary in a dilution of 1:48. In preliminary tests, a dilution of 1:60 gave what was considered an excess of mildly positive reactions. A dilution of 1:75 of the antigen was used in the final complement fixation tests.

The actual titration was carried out according to the routine Wassermann procedure. Dilutions of the patient's serum were made in physiological saline. The titration figures

[5] Levine, M. G., Hoyt, R. E., and Peterson, J. E., *J. Clin. Invest.*, 1947, **26**, 281.

presented in Tables I and II are based on serum in saline dilutions. To each tube was added 1 cc of guinea pig complement (2 units), and .5 cc of the 1:75 dilution of antigen. The whole was stored overnight in the refrigerator and then incubated for 10 minutes at 37°C. Following this, .5 cc of rabbit amboceptor (2 units) and .5 cc of a 2% sheep cell suspension were added. The tubes were then incubated at 37°C for 30 minutes and read for hemolysis according to the usual technic of measuring the degree of hemolysis by the symbols, 1+, 2+, 3+ and 4+, the last indicating no hemolysis. Proper controls were run for anticomplementary action of each ingredient of the test.

Results. A total of 534 patients' sera were tested for complement fixation. The individuals were suffering from a variety of diseases such as are to be seen in a consecutive series of applicants for admission to any hospital. Occasional tests showed a doubtful positive, 1+ or less in tube 1. These were not counted. All other positive reactions are listed in Table I. Fifteen patients, or 2.8% gave a positive reaction.

If we now examine the results obtained with sera from patients with clinically diagnosed rhinoscleroma, we find in 11 patients a positive reaction in each case and a relatively high titer of complement-fixing antibody.

From Table II we may conclude that the complement-fixation test is of value in the diagnosis of rhinoscleroma, but in addition it gives further evidence of a relationship of the organism described to the disease itself. Further work is in progress to determine if the sensitivity of the test is such as to permit its use in the diagnosis of early unrecognized scleroma.

Conclusion. A complement-fixation test for scleroma is described which offers evidence for the relationship between an organism described and the disease. The value of the test is indicated in diagnosing scleroma.

15870

Urobilinogen in Cerebrospinal Fluid.

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With the exception of references[1] to old observations by Mestrezat and by Milian on cerebrospinal urobilin and fluorescence, respectively, only the report by Creyx, Georget and Bonnel[2] on urobilin in spinal fluid in a case of Weil's disease was found in the literature. In the present investigation cerebrospinal fluids obtained at autopsy were tested with Ehrlich's reagent for urobilinogen and the results were compared with the reactions observed in urine and with Schlesinger's test for urobilin in the blood.

Technic. The *cerebrospinal fluid* was obtained by cisternal puncture and a red cell count was made to determine the degree of contamination with blood, after which the fluid was centrifuged. 2.5 cc of clear fluid were mixed in a small tube with 2 drops of 1% *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid[3] and the color viewed through the column of fluid against a white background. Normally a faint yellow tinge is observed which may become more intensely yellow if the concentration of urea[4] is high. In the presence of urobilinogen a pink

1 Cited in Greenfield, J. G., and Carmichael, E. A., *The Cerebrospinal Fluid in Clinical Diagnosis*, p. 197, Macmillan, 1925.

2 Creyx, M., Georget, F., and Bonnel, H., *J. de Méd. de Bordeaux*, 1935, **112**, 527.

3 Niemann, G., *Z. f. physiol. Chem.*, 1925, **146**, 182.

4 Naumann, H. N., *J. Lab. Clin. Med.*, 1938, **23**, 1127.

TABLE I.
Cerebrospinal Urobilinogen, Urobilin, and Bilirubin Compared to Bile Pigments in Blood and Urine.

Diagnosis, hr post-mortem	Cerebrospinal fluid			Blood			Urine			Other data
	Urobilinogen mg %	Urobilin	Bilirubin mg %	Red cells mm³	Bilirubin mg %	Urobilin	Urobilinogen mg %	Bilirubin		
1. Lung abscess, liver cirrhosis, intestinal hemorrhage. H.P.M. 8½	9.0	3+	0.4	5000	0.3	3+	27.5	±		—
2. Cholelithiasis, obstructive jaundice, pleural exudate, arteriosclerosis. H.P.M. 19	4.4	3+	<0.1	8000	4.5	2+	36.3	+		Fecal urobilinogen 1560 mg %
3. Encephalomalacia, pneumonia, fatty degeneration of heart, liver, kidneys. H.P.M. 14	1.8	2+	<0.1	400	0.4	1+	112.0	±		—
4. General paresis, malaria treatment, pneumonia. H.P.M. 10	1.4	1+	<0.1	1500	0.5	1+	104.0	±		—
5. Cardiac hypertrophy, pneumonia, subdural, subarachnoid hemorrhage. H.P.M. 13	±	±	5.5	1500	3.0	±	0.8	±		—
6. Arteriosclerosis, pulmonary infarct, subdural hemorrhage. H.P.M. 8	±	±	2.3	10800	0.8	±	0.5	±		—

color develops which shades into orange if the yellow color due to urea is also increased. The quantitative urobilinogen determination was performed by a modified Terwen method.[5] Schlesinger's zinc acetate-alcohol reagent was used for urobilin. When bilirubin was indicated by the icterus index, van den Bergh's reaction and Fouchet's test were applied. The quantitative bilirubin determination was performed according to Malloy and Evelyn.[6] *Blood* was obtained from the heart or its large vessels and was oxalated and centrifuged. The clear plasma, which contained some hemoglobin, was used for a qualitative urobilin test by a modification of Blankenhorn's procedure,[7] and for a determination of bilirubin according to van den Bergh. Urine was analyzed for bilirubin by the author's talc adsorption technic[5] and urinary and fecal urobilinogen were determined as indicated above.

Results. Of a total of 41 postmortem cerebrospinal fluids examined 4 gave a distinct pink reaction with Ehrlich's reagent, 11 gave a faint pink or orange color, and 26 were negative. The first 4 mentioned and 2 xanthochromic fluids were studied in greater detail.

As noted in Table I, urobilinogen ranged from traces to 9 mg % and the urobilin gave doubtful to strong fluorescence. There was a rough parallelism between cerebrospinal and urinary urobilinogen and plasma urobilin with increases in the first 4 cases while the increase in the last 2 cases was uniformly very slight or doubtful. The direct van den Bergh reaction in Case 1 was delayed positive, and negative in Cases 5 and 6. The Fouchet test was positive in Cases 1, 5 and 6 and doubtful in Cases 2, 3 and 4.

An interesting observation was the gradual appearance of a green fluorescence in the untreated cerebrospinal fluids of Cases 1 to 4

5 Naumann, H. N., *Bioch. J.*, 1936, **30**, 762 and 1021.

6 Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 480; Lepehne, G., *J. Lab. Clin. Med.*, 1942, **28**, 229.

7 Blankenhorn, M. C., *J. Biol. Chem.*, 1928, **80**, 477.

after being kept in the refrigerator for several days. This fluorescence was proportional in strength in the previously determined urobilinogen content, indicating the slow oxidation of the latter into urobilin which under the conditions of its solution in the cerebrospinal fluid exhibited a fluorescence greater than usual. This observation corresponds to that of Creyx, Georget and Bonnel[2] who found a fluorescence in the fresh spinal fluid of their case of Weil's disease, prompting them to establish the occurrence of urobilinorachia.

Comment. The origin of urobilinogenorachia may be explained by the diffusion of urobilinogen from the blood into the cerebrospinal fluid whenever the blood urobilinogen is sufficiently increased and the blood-cerebrospinal fluid barrier is disturbed. The conditions under which urobilinogen and bilirubin may pass the blood-cerebrospinal fluid barrier do not necessarily appear to coincide, as illustrated by Case 2 in which the cerebrospinal fluid contained 4.4 mg % urobilinogen and practically no bilirubin while the plasma bilirubin amounted to 4.5 mg %. On the other hand, Case 1 seems to indicate that diffusion of both pigments took place at a similar rate. As xanthochromic spinal fluids in jaundice are rare[8] factors other than liver damage can be assumed to be responsible for the increased barrier permeability, the most likely being infection.[9] The present cases showed severe pulmonary infection and in one instance malaria in addition.

Summary. Six cases of urobilinogenorachia have been presented in which the postmortem cerebrospinal fluid urobilinogen ranged from traces to 9 mg %.

8 Klein, N., and Szentmihalyi, S., *Dtsch. Arch. f. klin. Med.*, 1932, **173**, 234; Lickint, F., *Z. f. d. ges. Neurol. u. Psych.*, 1931, **136**, 291.

9 Malamud, W., and Rothschild, D., *Arch. Neurol. Psych.*, 1931, **24**, 348.

15871

Effect of Thiols on Mercurial Diuresis.

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The formation of stable complexes between mercury and thiols[1,2] led us to believe that the use of the latter compounds might yield further evidence of the mechanism of action of mercurial diuretics as well as the mechanism of the normal transport of water across the renal tubule. Peters[3] and his associates have presented ample evidence that many of the physiologic actions of the trivalent arsenicals may be explained by the reaction of the arsenical with essential sulfhydryl groups of certain enzymes, thereby inactivating the enzyme. Barron and Singer[4] have demonstrated that organic mercurials are also powerful inhibitors of some enzymes containing sulfhydryl groups.

This report concerns the effect of several compounds that contain sulfhydryl groups on mercurial diuresis.

Methods. The basal urine output of female dogs with inlying catheters was measured; the mercurial diuretic was then given intravenously, and the diuresis curves (Fig. 1) were obtained by recording 20-minute urine volumes over a period of about 3 hours.

1 Gilman, A., *Fed. Proc.*, 1946, **5**, 285.

2 Braun, H. A., Lusky, L. M., and Calvary, H. O., *J. Pharmacol.*, Suppl., 1946, **87**, 119.

3 Peters, R. A., Stocken, L. A., and Thompson, H. S., *Nature*, 1945, **156**, 616.

4 Barron, E. S. G., and Singer, T. P., *J. Biol. Chem.*, 1945, **157**, 221.

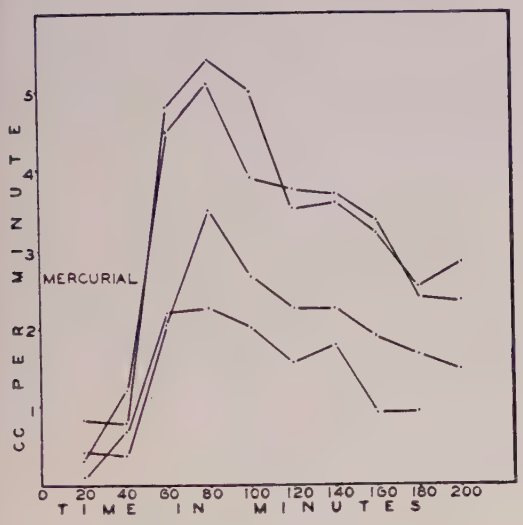

FIG. 1.
Mercuhydrin diuresis curves.

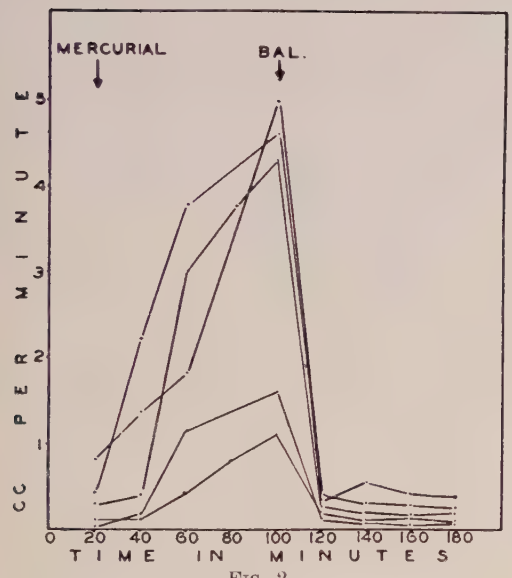

FIG. 2.
Antagonism of mercurial diuresis by Bal.

1-(methoxy-oxymercuri-propyl) - 3 - succinyl urea* (Mercuhydrin) and Mersalyl, U.S.P., were used. With these agents, in a dosage of 10 mg per kg given intravenously, maximal diuresis appears in about one hour in dogs. Diuresis usually continues for many hours. In another group of dogs, one of the thiols was administered intravenously during maximal diuresis.

Results. Fig. 2 shows that 2,3-dimercaptopropanol (Bal) immediately returns the urine output to the prediuretic level. The effect of Bal is permanent, although another dose of mercurial will again produce diuresis which, in turn, may be abolished by Bal. If Bal is injected before, or soon after, the mercurial, no diuresis develops. Thioglycolic acid has a similar action, but the effective dose is much larger. Methylene blue and glutathione temporarily inhibit mercurial diuresis while cysteine is ineffective in the dosage employed. Hematuria was sometimes observed when Bal and thioglycolic acid were administered after the mercurial diuretics, indicating that the thiol-mercury complexes may produce kidney damage. None of the thiols had any influence on water diuresis or normal urine output, but the diuresis produced by mercuric chloride was rapidly abolished by Bal.

Discussion. The demonstration that certain thiols effectively counteract the acute toxic effects of organic mercurial compounds[5] and abolish the diuretic action, furnishes additional evidence that both toxicity and diuresis from these agents are due to the liberation of free mercury. Bal, the most effective antagonist, forms a stable complex with mercury.

A study of kidney enzymes inhibited by the mercurial diuretics should yield information on the enzyme systems concerned in the reabsorption of water by the renal tubules. We are investigating this possibility.

Summary. The diuretic action of organic mercurials or inorganic mercury compounds is prevented or abolished by certain thiols. Of the compounds tested, Bal was the most effective, followed by thioglycolic acid, glutathione, and methylene blue in decreasing order of activity. Cysteine had very little effect.

* Kindly supplied by Dr. E. L. Foreman of the Lakeside Laboratories.

5 Long, W. K., and Farah, A., *Science*, 1946, **104**, 220.

15872

Effect of Nicotinic Acid on Myocardial Systole, Coronary Flow, and Arrhythmias of Isolated Heart.

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The known role of nicotinic acid in carbohydrate metabolism, on which, of course, contraction of muscle largely depends, suggests that this material might have a favorable effect on various disturbances of myocardial function. Briefly to review the chemical processes accompanying contraction, it should be recalled that Lundsgaard[1] showed that the initial energy for the contractile mechanism is derived from the breakdown of phosphocreatine (Eggleton and Eggleton;[2] Fiske and Subbarow[3]) into phosphoric acid and creatine. In order for muscular activity to continue, phosphocreatine must be regenerated; and this resynthesis is accomplished by energy derived from the metabolism of carbohydrate. By a series of enzymatic changes (reviewed by Best and Taylor[4]), glucose is broken down into 2 triose compounds, one of which is 3-phosphoglyceraldehyde. It is with the oxidation of this triose that nicotinamide is concerned; this amide constitutes the prosthetic group of diphosphopyridine nucleotide, a coenzyme which, in the presence of a specific protein, accepts hydrogen from 3-phosphoglyceraldehyde and thus accomplishes its oxidation into phosphoglyceric acid. The latter then undergoes enzymatic rearrangements to form pyruvic acid (von Euler *et al.*[5])

From this point, the fate of the reduced pyridine nucleotide and of pyruvic acid varies depending on the presence or absence of oxygen. Under aerobic conditions the pyruvic acid is oxidized through the medium of thiamine and flavoprotein-cytochrome systems to carbon dioxide and water. At the same time, the reduced pyridine nucleotide is rapidly reoxidized and thus may participate over and over again in the primary oxidation of the original triose.

In the absence of oxygen, on the other hand, the direct reoxidation of the reduced pyridine nucleotide by oxygen is no longer possible. At the same time and for the same reason, pyruvic acid also cannot be removed by oxidation; and so, under these anaerobic conditions, the 2 interact, yielding lactic acid and the oxidized form of the pyridine nucleotide. Thus regenerated, the coenzyme is again available to act as a hydrogen acceptor and the anaerobic breakdown of carbohydrate can proceed until equilibrium conditions or acid formation call a halt to the process. (Reviewed by Ball[6]). Though this anaerobic mechanism represents incomplete combustion and succeeds in liberating only a part of the total energy available were oxygen present, its role in muscular contraction is crucial. Its effectiveness, moreover, obviously depends on an adequate supply of

* Laboratory facilities were generously provided by the departments of pharmacology in the Medical Schools of the University of Texas, Johns Hopkins University, and Duke University.

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1 Lundsgaard, E., *Biochem. Z.*, 1930, **217**, 162; 1930, **227**, 51; *Ann. Rev. Biochem.*, 1938, **7**, 377.

2 Eggleton, P., and Eggleton, G. P., *Biochem. J.*, 1927, **21**, 190; *J. Physiol.*, 1927, **63**, 155; 1928, **65**, 15.

3 Fiske, C. H., and Subbarow, Y., *Science*, 1927, **65**, 401; *ibid.*, 1928, **67**, 169; *J. Biol. Chem.*, 1929, **8**, 629.

4 Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, Baltimore, The Williams and Wilkins Co., 2nd edition, 1939, 984.

5 von Euler, H., Adler, E., Günther, G., and Hellström, H., *Z. physiol. Chem.*, 1937, **245**, 217.

6 Ball, E. G., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 253.

nicotinamide in the oxidized rather than the reduced form.

Glycolysis in cardiac muscle proceeds along lines similar to those of skeletal muscle. (Ochoa[7]). Nicotinamide is therefore as necessary for cardiac activity as it is for contraction of striated voluntary muscle; and that its deficiency might interfere with the function of the heart is to be expected. The observations of Feil,[8] Mainzer and Krause,[9] and Rachmilewitz and Braun[10] are in line with this theoretical implication. These authors detected electrocardiographic changes in from 50 to 75% of patients with pellagra, and in many instances were able rapidly to reverse the abnormalities by nicotinamide therapy. Were such changes confined simply to pellagrins, the usefulness of this form of treatment would be sharply circumscribed; but additional potentialities are opened by the discovery (Mann and Quastel[11]) that *damaged* tissues possess an agent which breaks down cozymase at a rapid rate. This may take place under strictly anaerobic conditions, and is due, according to the interpretation of Mann and Quastel, to hydrolysis of the coenzymes by a nucleotidase. The addition of nicotinamide to such inactivated systems results in a marked stimulation of oxygen uptake. It is known (Kohn;[12] Axelrod *et al.*[13]) that the administration of nicotinic acid is followed by an increase in the concentration of the pyridine nucleotides, and the possibility therefore arises that nicotinic acid or its amide might prove efficacious under a variety of conditions in which cardiac tissue has been damaged and the pyridine nucleotides consequently inactivated.

Direct investigations of the action of nicotinic acid and its amide on the heart have been few. Scaffidi and d'Agostino[14] concluded that the dilute acid has a vasodilator action on the coronary circulation but that stronger solutions cause vasoconstriction. They do not comment on the action of the drug on the myocardium itself, nor, indeed, is it clear from their description that the observed changes might not have been due to poor control of hydrogen-ion concentration.

These limited data on nicotinic acid itself are somewhat supplemented by the numerous studies that have been conducted on coramine, which is the diethylamide of nicotinic acid. In certain respects at least, coramine duplicates the physiological action of the latter (Smith[15]). Uhlmann[16] showed that coramine directly stimulates the perfused heart poisoned with chloral hydrate or choline. Using Starling's heart-lung preparations (which are essentially *normal* hearts), Leyko[17] found that coronary flow is increased, regardless of the state of contraction, which he found to be unaltered by small doses but depressed by stronger concentrations of coramine. By the same technic, Mezey[18] made the important distinction between its action on the normal heart as contrasted with that on the insufficient or poisoned myocardium. In the former, the effects of coramine are negligible, there being an increase in diastole with resultant increase in stroke volume; but a concomitant decrease in frequency causes the minute volume to remain the same. In contrast, in the *insufficient* heart there occur reduction of elevated venous pressure as measured in the right heart, increase in diastole with consequent lessening of venous stasis, rise in arterial pressure, and an in-

7 Ochoa, S., *Biochem. Z.*, 1937, **290**, 62.

8 Feil, H., *Am. Heart J.*, 1936, **11**, 173.

9 Mainzer, F., and Krause, M., *Brit. Heart J.*, 1940, **2**, 85.

10 Rachmilewitz, M., and Braun, K., *Am. Heart J.*, 1944, **27**, 203.

11 Mann, P. J. G., and Quastel, J. H., *Nature*, 1941, **147**, 326; *Biochem. J.*, 1941, **35**, 502.

12 Kohn, H. I., *Biochem. J.*, 1938, **32**, 2075.

13 Axelrod, A. E., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1939, **131**, 85; Axelrod, A. E., Gordon, E. S., and Elvehjem, C. A., *Am. J. Med. Sc.*, 1940, **199**, 697.

14 Scaffidi, V., Jr., and d'Agostino, L., *Riv. pat. sper.*, 1939, **22**, 437.

15 Smith, David T., Margolis, George, and Margolis, Lester H., *J. Pharmacol. and Exp. Therap.*, 1940, **68**, No. 4, 458.

16 Uhlmann, F., *Z. ges. exp. Med.*, 1924, **43**, 556.

17 Leyko, E., *J. Pharmacol. and Exp. Therap.*, 1930, **38**, 31.

18 Mezey, K., *Klin. Woch.*, 1935, **14**, 1176; *Arch. exp. Path. u. Pharmakol.*, 1935, **177**, 235.

crease in minute volume. Coronary dilatation has been noted by Greene[19] and Stoland and Ginsberg;[20] and Cowan[21] has shown that increase in voltage of the electrocardiogram, elevations of the T-wave in leads I and II, and correction of preexisting hypotension may follow the administration of coramine.

Suggestive as these numerous observations are, it seemed worth while to investigate the action of nicotinic acid on the perfused heart, and to note especially its effect in those cases in which the myocardium was unable to function normally.

Experimental. Methods. Rabbits were killed by a quick blow on the head, and the heart was removed rapidly and mounted on a perfusion apparatus in such way that the perfusion fluid entered the aorta above the valves and was thus forced into the coronary vessels. The effluent was measured either in a graduate or in a small, subjacent pan so arranged that each 5 cc of fluid would cause the pan to trip, empty itself, and close an electrical circuit activating a signal on the kymograph. The temperature of the perfusion fluid was kept constant at 37°C by means of a water jacket regulated by a Bratton thermostat.[22] A modified Ringer-Locke solution was employed as a perfusion fluid, each liter containing the following: sodium chloride, 21.7 cc of a 30% solution; sodium bicarbonate, 23.0 cc of an 8% solution; potassium chloride, 4.7 cc of a 9% solution; anhydrous calcium chloride, 4.8 cc of a 5% solution. When a mixture of 5% carbon dioxide and 95% oxygen was bubbled through this solution, the pH remained constant at 7.4, and the buffering capacity was such that it would tolerate the addition of nicotinic acid in a concentration as high as 1:100,000 without change of hydrogen-ion concentration. Small hooks were inserted into the epicardium on opposite sides of the heart, one being fixed and the other connected by means of fine silk to a heart lever, the excursions of which were recorded kymographically.

TABLE I.
Effect of Nicotinic Acid on Heart Rate and Coronary Flow.

Type of perfusion fluid	Pulse	Perfusion rate in cc per min
Ringer's	52	2.7
Nicotinic Acid 1:1,000,000	76	10.8
Ringer's	74	8.2
Nicotinic Acid 1:500,000	84	8.3
Ringer's	72	5.7
Nicotinic Acid 1:100,000	72	5.0
Ringer's	36	2.5
Nicotinic Acid, 1:100,000	32	2.9

Forty experiments were performed according to the method outlined, and nicotinic acid was employed in concentrations ranging from 1:100,000 to 1:2,000,000. Nicotinic acid amide was used in 6 of the experiments.

Results. Effect on Heart Rate. The effect of nicotinic acid on the heart rate was not striking in any instance. In two-thirds of the 40 experiments, the rate was increased from an initial average of 64 to an average of 76. In 2 cases, there was no change. These alterations apparently were not significant, for they did not exceed the variations in rate observed from time to time when Ringer's solution alone was used as the perfusion fluid.

Effect on Rate of Coronary Flow. The rate of flow of the perfusion fluid through the coronary vessels was increased in 31 preparations, decreased in 3 and unaffected in 6 instances. A typical protocol of this effect is shown in Table I. The magnitude of increase was from 1½-fold to as much as 10-fold. In all except 3 cases, this augmentation of coronary flow was accompanied by an increase in the amplitude of the myocardial excursion or by a moderate increase in heart rate. The increased flow, therefore, cannot be interpreted as necessarily implying that dilatation of the coronary vessels had occurred; for the improved action of the heart could have been sufficient to account for the observed increase in coronary flow. Moreover, as indicated in Table I, the change in rate of flow in general was observed only once in a given preparation, subsequent applications of nicotinic acid to the same preparation being without effect.

Effect on Amplitude of Cardiac Excursion.

19 Greene, C. W., *J. Pharmacol. and Exp. Therap.*, 1936, **57**, 98.

20 Stoland, O. O., and Ginsberg, A. M., *J. Pharmacol. and Exp. Therap.*, 1937, **60**, 396.

21 Cowan, J. H., *Am. J. Med. Sc.*, 1937, **193**, 673.

22 Bratton, A. C., *Science*, 1939, **89**, 589.

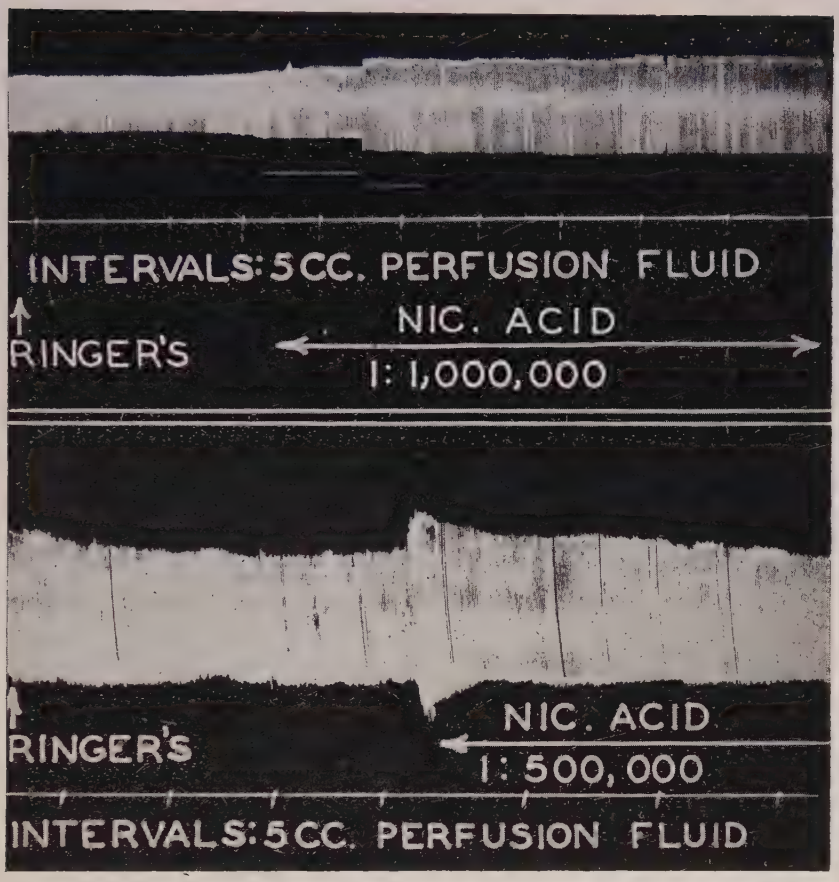

FIG. 1.
Effect of nicotinic acid on the amplitude of a poorly functioning heart.

In only 8 instances among the 40 experiments did nicotinic acid fail to augment the excursion of the cardiac muscle. This effect is shown graphically in Fig. 1, 2, and 3. As indicated in these kymographic tracings, this increase in amplitude was due principally to a more effective systole, there being only slightly greater diastolic relaxation than was observed with Ringer's solution alone.

In approximately three-fourths of the cases, this effect on systole was observed only once in a given preparation. When Ringer's solution was reapplied there was, of course, a slow and gradual decrease in cardiac excursion characteristic of preparations of this kind. Reapplication of nicotinic acid in such instances stimulated systole repeatedly in only about one-fourth of the cases. The most striking example of this recurring effect is illustrated in Fig. 2. This preparation varies from the usual in that 1 g glucose has been added to each liter of Ringer's solution.

Effect on Arrhythmia. One of the most impressive observations in the experiments was the abolition of certain arrhythmias by nicotinic acid. Spontaneous partial heart block was seen in 3 cases. The application of nicotinic acid in 2 of these immediately remedied the condition, while it was without effect in the third instance.

In 2 cases, ventricular standstill developed, apparently incident to technical delay in removing the heart from the chest cavity and mounting on the perfusion apparatus. In both instances, nicotinic acid was immediately effective in stimulating the myocardium to normal action (Fig. 4).

Four cases of spontaneous ventricular

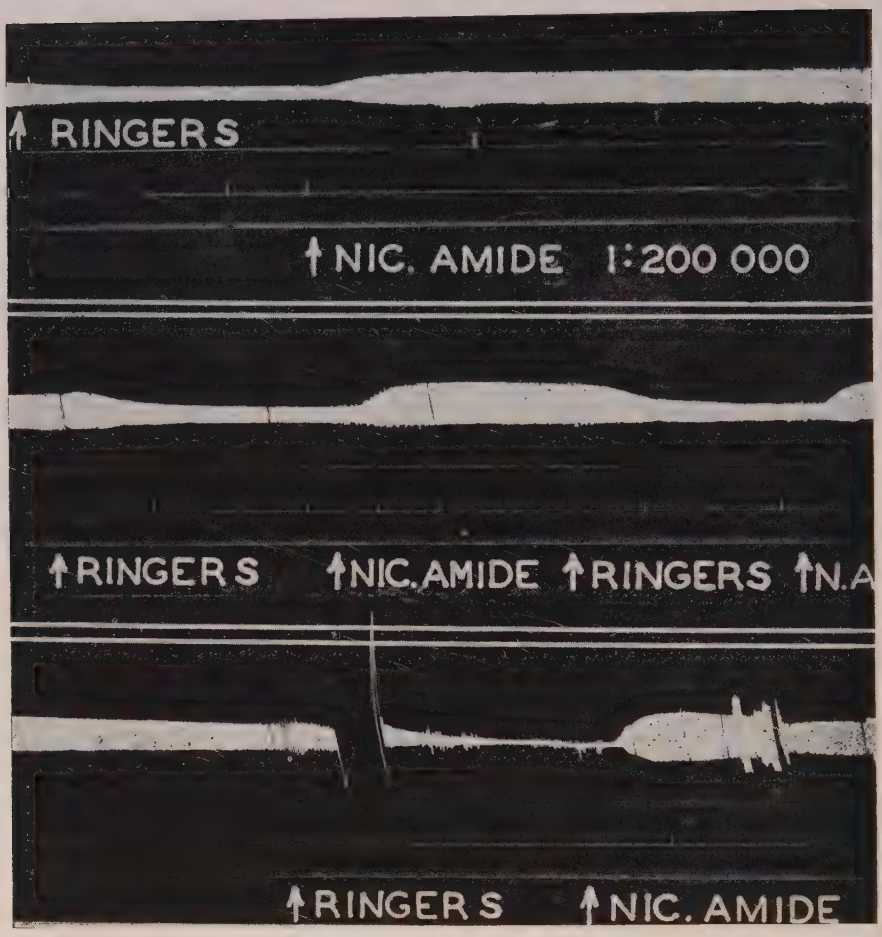

FIG. 2.
Repeated myocardial stimulation by nicotinamide. One percent glucose was incorporated in the perfusion fluid.

fibrillation were encountered in this series of experiments. Two of these were abolished by nicotinic acid (Fig. 5), while the other 2 were not affected. In one of these latter, both the fibrillation and its failure to respond favorably to nicotinic acid were probably explainable on the basis of thrombi which, it was discovered, had developed in major branches of the coronary arteries. Since the incidence of this abnormality as a spontaneous phenomenon is low, attempts were made in 2 preparations to reproduce the condition by electrical stimulation. In one of these experiments, electrical stimulation applied when the perfusion fluid contained 1:200,000 nicotinamide was capable of producing only very transient runs of fibrillation. Electrical stimulation in the absence of nicotinamide resulted in protracted fibrillation; reapplication of nicotinamide was followed promptly by the disappearance of this arrhythmia. In the other instance, nicotinamide in dilutions of 1:500,000 and 1:50,000 failed to abolish the abnormality.

Discussion. These beneficial effects of nicotinic acid on the failing myocardium demand an appraisal of the design and execution of our experiments in order to determine, if possible, the fundamental cause of the disturbances which proved remediable by nicotinic acid. Obviously, the abnormalities cannot be ascribed to a primary deficiency of this vitamin in the original preparation. One is forced, therefore, to search for factors

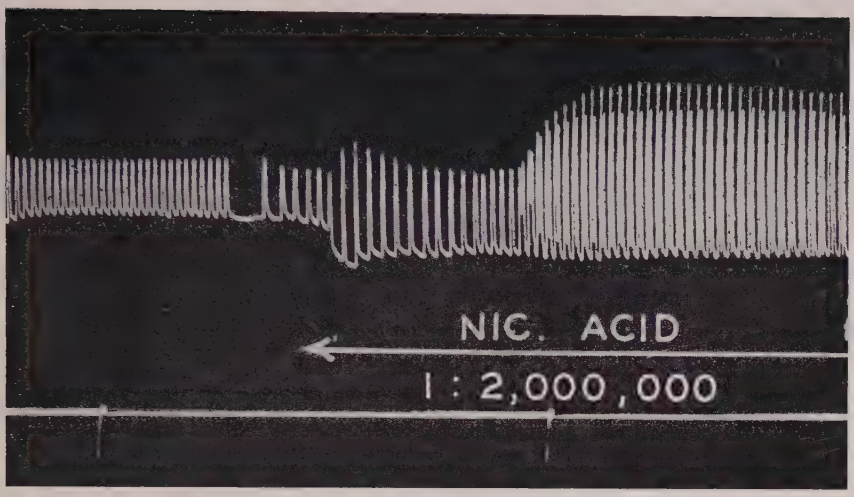

FIG. 3.
Effect of nicotinic acid on the dying heart. This preparation was 2 hours old.

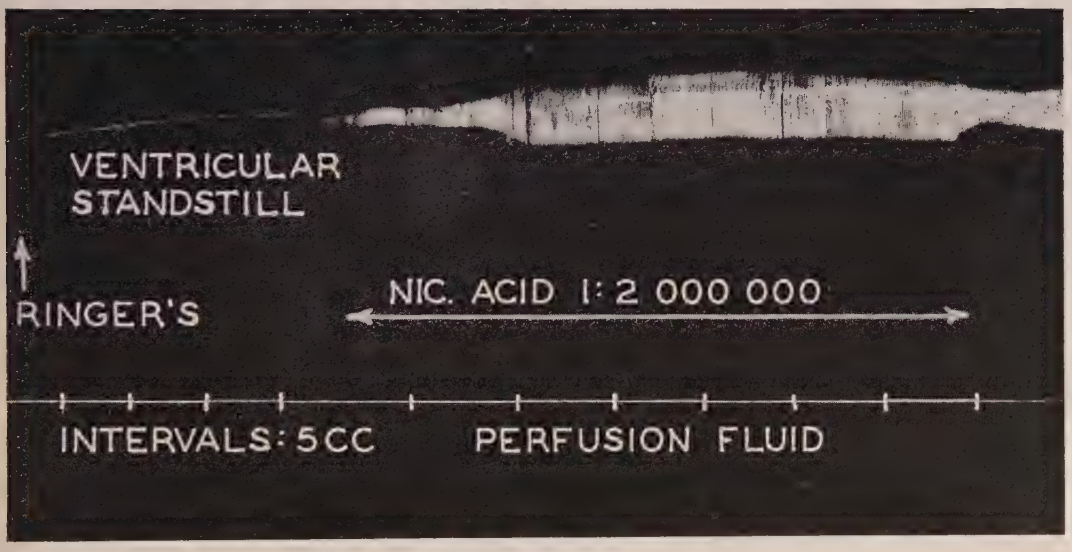

FIG. 4.
Effect of nicotinic acid on ventricular standstill.

that might lead to a temporary depletion or inactivation of diphosphopyridine nucleotide, of which nicotinamide is the prosthetic group.

On the basis of established biochemical principles 3 such factors come to mind: (1) Under the conditions of our experiments the heart was deriving its energy largely if not exclusively from carbohydrate. Sydenstricker[23] has stated that when carbohydrate is the principal source of energy, the pyridine nucleotides apparently are used up at a greatly increased rate. This circumstance, then, may have contributed to impairment of myocardial function. (2) The discovery of Mann and Quastel[11] noted above is of possible significance. It is conceivable, on this basis, that manipulative trauma induced the formation of a nucleotidase which could have accounted for hydrolysis of coenzyme with resultant cessation of glycolysis. (3) The

[23] Sydenstricker, V. P., *Arch. Int. Med.*, 1941, **67**, 746.

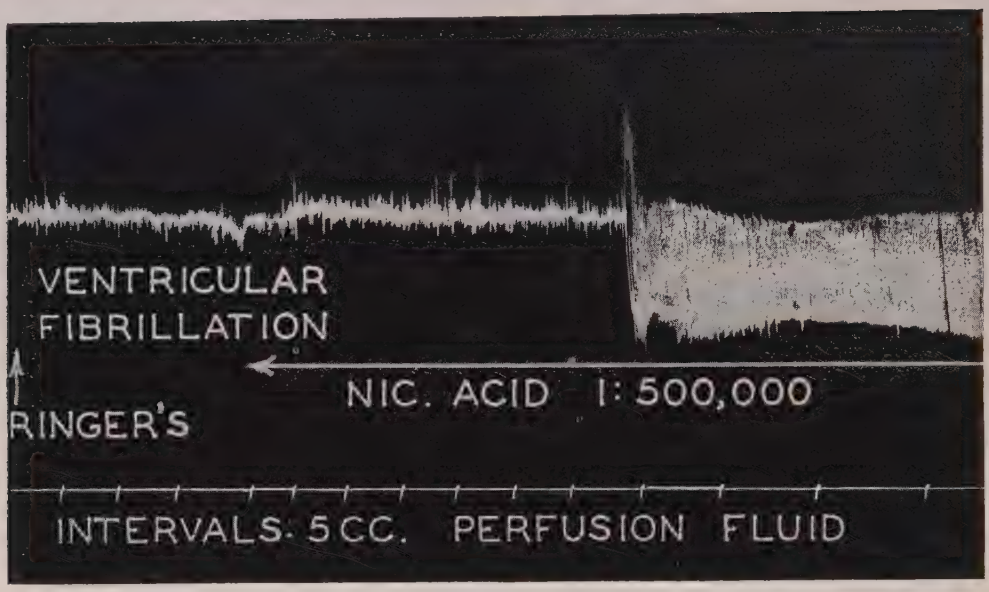

FIG. 5.
Effect of nicotinic acid on ventricular fibrillation.

most plausible explanation of the observed phenomena involves a consideration of the consequences of the temporary anoxia incident to technical delay in mounting the heart on the perfusion apparatus. It was held formerly (Meyerhof;[24] Warburg[25]) that the energy exchanges involved in enzymatic cellular respiration were entirely independent of oxygen tension and that they proceeded normally so long as the smallest amount of oxygen was present. Kempner,[26] however, was able to disprove this concept. Working with a variety of animal and bacterial cells, as well as with cell-free plasma, Kempner established definitely that if the oxygen tension decreases to very low levels, cellular respiration declines in rate and changes qualitatively, as evidenced by a marked fall in the respiratory quotient. The region of oxygen tension in which these changes are manifest, moreover, is above that at which anaerobic glycolysis begins. From what is known of the mechanisms by which cellular oxidation is mediated, this failure of cellular respiration seems to be due to the fact that the lack of oxygen impedes the rate at which the reduced catalysts are reoxidized. That cardiac function should suffer under the conditions of our experiments is therefore not surprising.

If, as seems entirely probable, the observed disturbances of cardiac function were actually due to inactivation or depletion of the pyridine nucleotides, it seems reasonable to believe that these temporary deficiencies are correctable by the addition of nicotinic acid or its amide to the perfusion fluid. Thus replenished, adequate coenzyme would be available for the resumption of anaerobic glycolysis, equilibrium between pyridine nucleotides and the other respiratory enzymes would be reestablished, and the aerobic phases of oxidation could then proceed in normal and orderly fashion.

Summary. Experiments designed to in-

[24] Gildemeister, M., and others, editors, Monographien aus dem Gesamtgebiet der Physiologie der Pflanzen und der Tiere. Herausgegeben von M. Gildemeister, R. Goldschmidt, C. Neuberg, J. Parnas und W. Ruhland. Band XXII: Die chemischen Vorgänge in Muskel und ihr Zusammenhang mit Arbeitsleistung und Wärmebildung, von Otto Meyerhof. Berlin, 1930, Julius Springer.

[25] Warburg, O., and Kubowitz, F., *Biochem. Z.*, 1929, **214**, 4.

[26] Kempner, W., *Cellular and Comp. Physiol.*, 1937, **10**, 339; *Cold Spring Harbor Symposia on Quantitative Biology*, 1939, **7**, 269.

vestigate the action of nicotinic acid on the isolated rabbit's heart are reported. Dilutions of from 1:100,000 to 1:2,000,000 were employed, and careful attention was paid to constancy of temperature and hydrogen-ion concentration.

It was found that the effect of nicotinic acid on well functioning hearts is insignificant. In the case of failure of the myocardium, however, this pyridine derivative causes a marked increase in amplitude of the cardiac excursion, reversal of abnormal rhythms (including partial heart block, ventricular fibrillation, and ventricular standstill), and at times a considerable augmentation of coronary flow. In no case does it appear to have an unfavorable influence.

The explanation of these effects may lie in the role played by nicotinic acid in carbohydrate metabolism. The idea is advanced that the observed disturbances of myocardial action are due to inactivation or depletion of the pyridine nucleotides by the anoxia incident to experimental technic; and that restoration of normal function probably depends on correction of this temporary and reversible deficiency by the addition of nicotinic acid to the perfusion fluid.

15873

Inactivation of Influenza Virus with Sulfur and Nitrogen Mustards.

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It has been demonstrated that sulfur and nitrogen mustards react chemically with a variety of proteins and enzymes of biological importance, and in so doing alter their physical properties and physiological functions. Presumably it is by such a mechanism of action that mustards abolish the infectivity of the nucleoprotein plant viruses causing tobacco mosaic and bushy stunt disease.[1] Recently, Tenbroeck and Herriott[2] have shown that the animal viruses of eastern equine encephalomyelitis, fixed rabies and hog cholera also may be rendered noninfectious by treatment with sulfur mustard without, however, altering their antigenicity. These mustard-inactivated viruses provoked a specific, protective immune response when injected into animals, suggesting the practical application of this method for the preparation of killed viral vaccines.

The present report deals with an investigation of the effects of sulfur mustard, bis-betachloroethyl sulfide (HS), and nitrogen mustards, bis- and tris-betachloroethyl amine (HN), on influenza virus. It will be shown that both types of mustard destroy the ability of this virus to infect susceptible animals, under appropriate conditions, but that high concentrations of these substances fail to alter its hemagglutinative properties.

Experimental. Effect of HS on Infectivity of Influenza Virus. The influenza virus employed was the PR8 strain in mouse lung passage. Dilutions of the virus in nutrient broth were made from 10% suspensions of infected lung, and the LD_{50} was found to be approximately .05 cc intranasally of a 10^{-6} dilution.

An approximately saturated solution of HS was prepared by adding 0.1 cc of HS to 50 cc of ice-cold 0.85% saline in a 250 cc stoppered flask, shaking vigorously by hand for one minute, and then placing the mixture in the refrigerator for 5 minutes to allow the undissolved residue of HS to separate and settle on the bottom of the flask. The assumption was made that the concentration of HS in the supernatant saline approached

1 Gilman, A., and Philips, F. S., *Science*, 1946, **103**, 409.

2 Tenbroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 271.

6×10^{-3} M, according to published data.[3] The saline saturated with HS was mixed with an equal volume of virus diluted 10^{-2}; similar mixtures of virus were made with HS saturated saline diluted 1-5, and with saline alone. The final concentrations of HS were therefore half-saturated and 10th-saturated, while the final concentration of the virus in each mixture was 5×10^{-3}. The preparations were kept at 4°C for 3 hours, and then each was inoculated into 5 mice by instilling .05 cc intranasally under ether anesthesia. The animals were observed for a period of 10 days, at the end of which time the survivors were sacrificed and examined.

All of the control animals died between the 5th and 8th days after inoculation and in every instance their lungs showed complete consolidation. The mice inoculated with the virus exposed to HS at 10th-saturation also were infected, although the disease was slightly less rapid and intense than in the controls; 4 mice died from 7 to 10 days after inoculation and one mouse survived. The lungs of the survivor showed approximately 60% consolidation. In sharp contrast to these findings, all of the mice inoculated with virus exposed to half-saturated HS survived 10 days and at autopsy their lungs appeared completely normal.

Effect of HN on Infectivity of Influenza Virus. Three nitrogen mustards were tested for their ability to inactivate influenza virus: ethyl (HN1) and methyl (HN2) bis (beta chloroethyl) amine hydrochloride, and tris (beta chloroethyl) amine hydrochloride (HN3). Each was dissolved in cold 0.85% saline containing .05 molar $NaHCO_3$ (pH 8.0) to give a concentration of 1 mg per cc. Immediately after preparation, the solutions were mixed with equal volumes of 10^{-2} mouse passage influenza virus (PR8) as previously described, and a virus control was made with bicarbonate-saline alone. The mixtures were kept at 4°C for 2 hours, and .05 cc of each preparation then was inoculated intranasally into 5 etherized mice. The animals were observed for 10 days, and survivors were sacrificed and examined at the end of that period.

All of the control mice died 5 or 6 days after inoculation and presented typical lung lesions. Of the mice inoculated with virus-nitrogen mustard mixtures, all receiving HN1 and 2 receiving HN2 also died. Four of these 7 deaths occurred between the 2nd and 4th day after inoculation, before any of the controls succumbed, and the others died on the 5th, 8th, and 9th days, respectively. The lungs of 4 of the dead animals showed only slight to moderate congestive changes, while the lungs of the remaining 3 mice appeared similar to those of the controls. However, the intranasal passage of 10^{-1} suspensions of these lungs to normal mice produced neither death nor pulmonary lesions, so it would seem possible that the effects observed could be attributed to the toxic action of HN1 and HN2 rather than to influenza virus.

Three of the mice that received virus treated with HN2 and all of those that received virus treated with HN3 survived 10 days, and at autopsy their lungs appeared normal.

Effect of HS and HN on the Hemagglutinative Property of Influenza Virus. A saturated solution of HS and solutions of the 3 HN compounds containing 1 mg per cc were prepared as before. These solutions were mixed with equal volumes of PR8 influenza virus in the form of infected chick embryonic chorioallantoic fluid. After standing for 3 hours at 4°C serial doubling dilutions of the mixtures were made in tubes containing 0.5 cc of saline; 0.5 cc of a 1.5% suspension of adult chicken erythrocytes then was added to each tube. An appropriate control was included. The tubes were observed at room temperature over a period of 2 hours and the agglutinative titers were determined by the sedimentation pattern method.[4]

The results of this test are shown in Table I. It is apparent that neither HS nor HN, in the concentrations employed, affected the ability of the virus to agglutinate the erythrocytes.

HN Treatment of Mice Infected with Influenza Virus. Three series of mice were in-

[3] Herriott, R. M., Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, 1946, **30**, 185.

[4] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE I.
Effect of HS and HN on Agglutination of Chicken Erythrocytes by Influenza Virus.

	Reciprocal of dilutions*								
	4	8	16	32	64	128	256	512	1024
Virus + HS half-saturated	++++	++++	++++	++++	++++	+++	++	+	+
Virus + HN1 .5 mg per cc	++++	++++	++++	++++	++++	+++	++	+	0
Virus + HN2 .5 mg per cc	++++	++++	++++	++++	++++	+++	+++	+	+
Virus + HN3 .5 mg per cc	++++	++++	++++	++++	++++	+++	++	+	+
Virus + saline	++++	++++	++++	++++	++++	+++	++	+	+

* 0 to ++++ = degree of sedimentation and pattern of erythrocytes after 2 hours at 25°C.

oculated intranasally with PR8 influenza virus 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. In each series 5 mice were inoculated with each dilution of virus. Two series of these animals were treated with HN2 by the daily intraperitoneal injection of 0.5 mg per kg beginning 2 days before inoculation in one series and immediately after inoculation in the other. The third series served as controls. Therapy with HN2 did not modify the infection, and details will not be presented.

Discussion. The results of this study indicate that both sulfur and nitrogen mustards in relatively high concentration are able quickly to destroy the infectivity of influenza virus *in vitro*. Similar concentrations of these agents appear to have no effect upon the hemagglutinative properties of the virus. These findings emphasize once more the essential distinction between the power of the virus to produce infection and its ability to agglutinate erythrocytes of certain species. They suggest also that the loss of infectivity following exposure to mustards may not be accompanied by a comparable loss of antigenicity, although further work is necessary to establish more direct proof of this point.

One drawback in the use of sulfur mustard for the inactivation of viruses is its immiscibility with aqueous solutions and the consequent difficulty of predicting the amount dissolved under varying conditions of time, temperature, rate of mixing and so forth. On the other hand, the nitrogen mustards in the form of their hydrochloride salts are crystalline and are readily soluble in water, so that solutions of known concentration can be made accurately. A wider exploration of the possibilities of the nitrogen mustards as inactivating agents for viruses is indicated.

Conclusions. Influenza virus was rendered noninfectious for mice by *in vitro* exposure to relatively high concentrations of sulfur and nitrogen mustards. Concentrations of the mustards that abolished infectivity did not reduce the ability of the virus to agglutinate erythrocytes. The parenteral administration of nitrogen mustard had no effect on the course of infection with influenza virus in mice.

15874 P

Degeneration and Necrosis of Neurones in Eighth Cranial Nuclei Caused by Streptomycin.

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Toxic effects of streptomycin on the eighth cranial nerve apparatus have been frequently noted.[1-3] but the pathogenesis of these disturbances has not yet been established.[1,2,4,5]

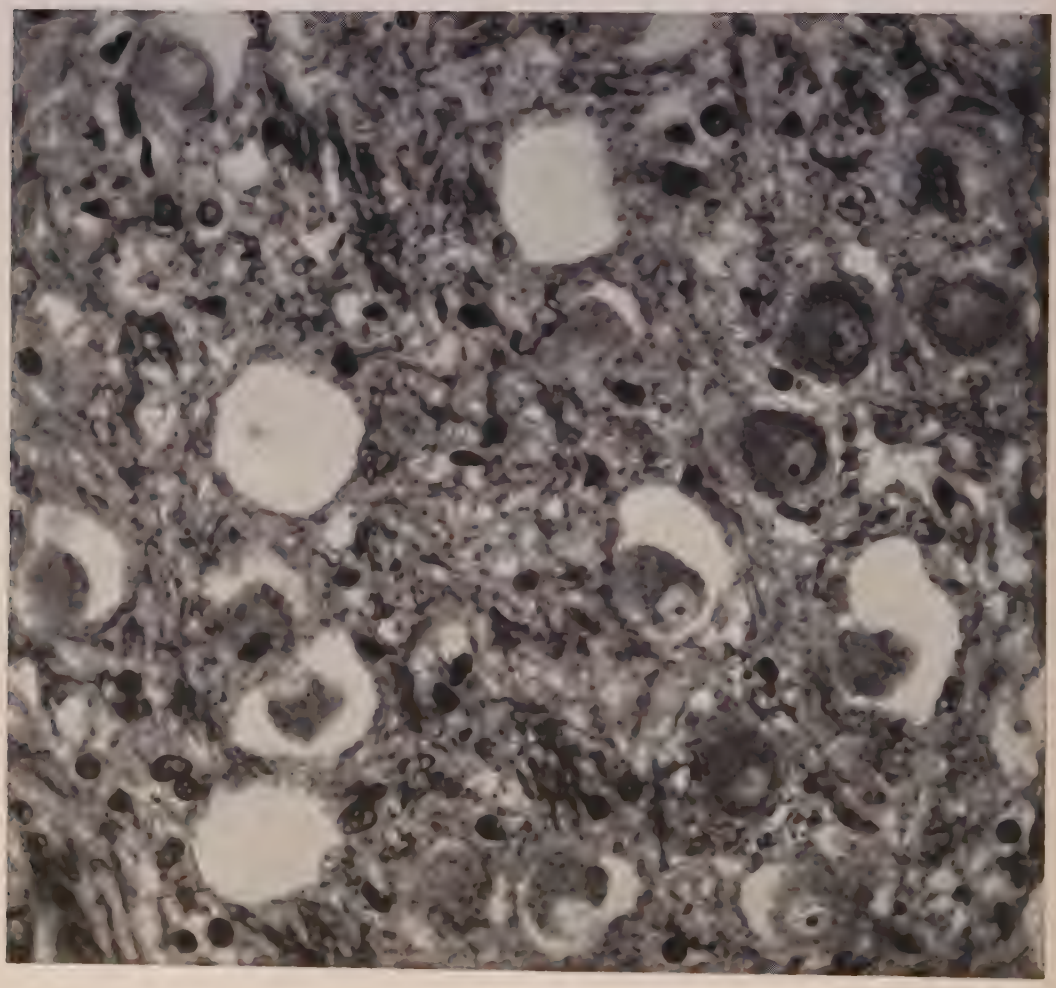

FIG. 1.
Patient No. 1, Masson trichrome stain, × 625: Group of cells in the ventral cochlear nucleus, showing various stages of liquefaction necrosis with dropping out of some cells. Identical changes were present in both inferior vestibular nuclei in this patient.

[1] Baggenstoss, A. H., Feldman, W. H., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1947, **55**, 54.

[2] Brown, H. A., and Hinshaw, H. C., *Proc. Staff Meet. Mayo Clin.*, 1946, **21**, 347.

[3] Farrington, R. F., Smith, H. H., Bunn, P. A., and McDermott, W., in press.

[4] Fowler, E. P., Jr., and Seligman, E., *J. Am. Med. Assn.*, 1947, **133**, 87.

[5] Keefer, C. S., Blake, F. G., Lockwood, J. S., Long, P. H., Marshall, E. K., Jr., and Wood, W. B.

TABLE I.

Summary of Clinical and Pathological Effects of Streptomycin on Eighth Nerve Nuclei in 5 Patients.

Patient No.	Days of streptomycin		Ventral cochlear nucleus		Inf. vestib. nucleus. Neuronal changes	Tb. meningitis	Other findings
	I.M.	I.T.	Deafness	Neuronal changes			
1	68	59	++++	+++	+++	+	0
2	154	27	+++	++	++	++	Tubercles in medulla
3	147	54	++++	+++	0	+++	Hydrocephalus
	94	94					
4	98	45	++++	++	0	++++	Softening in pons (unilateral), tuberculoma in cerebellum
5	61	0	++++	+	0	0	Tubercles in pons, nephritis
	106	0					
	5	0					

The patients were treated on the medical and pediatric pavilions of the New York Hospital as part of an investigation of the effects of streptomycin in tuberculosis.[6]

I.M. = Intramuscular streptomycin, approximately 3 g daily in adults—children proportionately less.

I.T. = Additional streptomycin given intrathecally.

Deafness was evaluated clinically in all patients, and serial audiograms were also done in patients 2, 4, and 5.[9]

The neuronal changes consisted of varying degrees of liquefaction necrosis, sometimes with dropping out of cells. See Fig. 1.

This preliminary report is based on the neuropathological findings in 5 human beings who became partially or completely deaf while receiving large amounts of streptomycin, and on the study of the eighth cranial nerve nuclei in 3 dogs given the drug experimentally.

Table I provides a summary of the findings in the 5 clinical cases. It is noteworthy that all of the patients died with tuberculosis and all manifested varying degrees of tuberculous involvement of the central nervous system, though there was no clinical evidence that the function of any of the cranial nerves except the eighth had been disturbed in any of the cases. In one case (patient No. 4) there was softening of a part of the basis of the pons immediately below the ventral cochlear nucleus, this change being unilateral whereas the degeneration and necrosis of neurones presumably due to streptomycin was bilateral.

To learn whether neuronal changes similar to those encountered in the 5 patients could be produced experimentally, 3 medium sized, adult, mongrel dogs were given 170 mg of highly purified streptomycin per kg of body weight during 12 hours each day in 4 equal doses intramuscularly, this being roughly equivalent to a 12 g dose for an adult human being.[10] All 3 dogs developed marked ataxia, weaving of the head, tail-chasing, and weakness following administration of the drug, the symptoms being markedly accentuated following the final dose each evening. An accurate appraisal of auditory acuity could not be made, but none of the animals became manifestly deaf. One of the dogs died on the 9th day with advanced, bilateral, necrotizing renal arteriolitis and glomerulitis; the

Jr., *J. Am. Med. Assn.*, 1946, **132**, 4, 70.

[6] McDermott, W., Muschenheim, C., and Ha S. J., in press.

[7] Molitor, H., Graessle, O. E., Kuna, S., Mu C. W., and Silber, R. H., *J. Pharm. and Therap.*, 1946, **86**, 151.

[8] Mushett, C. W., and Martland, H. S., *Arch. Path.*, 1946, **42**, 619.

[9] Moore, J. A., unpublished data.

[10] Engle, R. L., Jr., and Farrington, R. F., unpublished data.

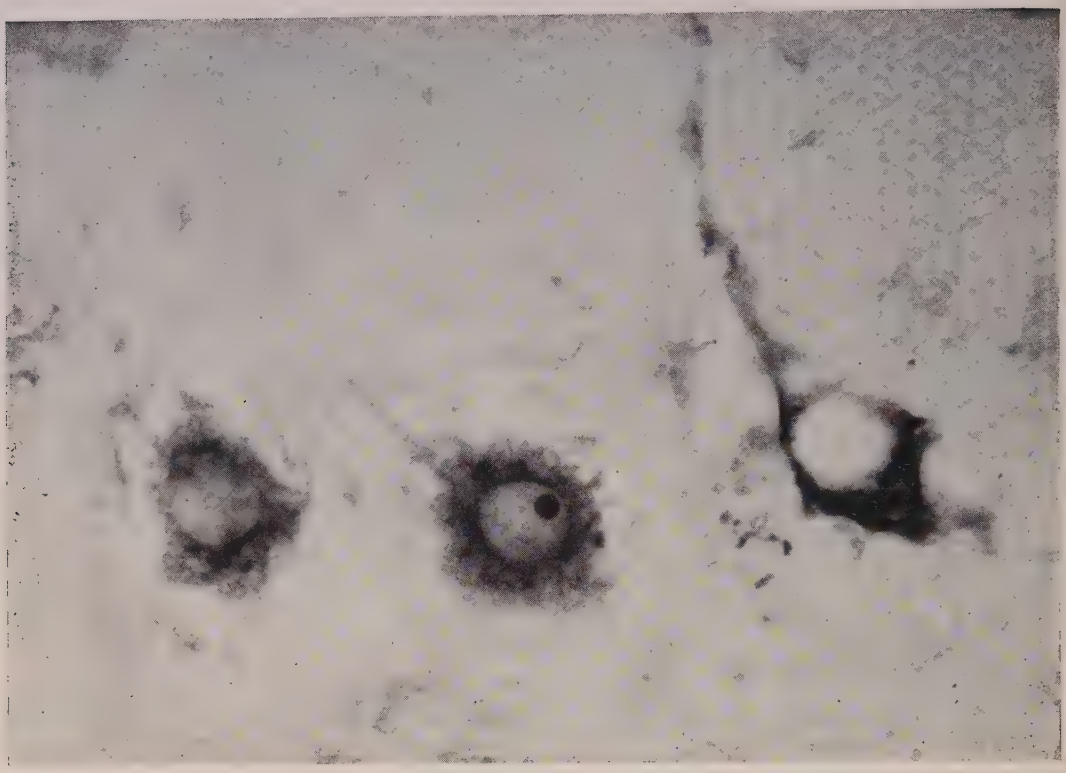

FIG. 2.
Dog No. 2, Nissl stain, × 1250: Group of cells in the ventral cochlear nucleus, showing marked liquefaction necrosis.

other 2 were sacrificed on the 28th day. Liquefaction necrosis was found bilaterally in the ventral cochlear nuclei in all 3 animals; it was advanced in one case (Fig. 2), and, in the dog that died early in the experiment with renal changes, was associated with a curious clumping of Nissl-like material in the majority of the neurones of these nuclei.

In summary, pathological changes were present in the neurones of the ventral cochlear nuclei in all 5 clinical cases and in all 3 experimental animals, although in 2 of the patients and in one of the animals these changes were noted in only a few cells. No lesions were noted in the dorsal cochlear nuclei. The vestibular nuclei generally showed no striking change, except for the inferior vestibular nucleus, which in 2 cases showed a similar marked liquefaction necrosis and dropping out of cells and hydropic degeneration. The superior vestibular nuclei were not definitely identified. Other cranial nerve nuclei were examined in every case, often in the same histological preparations as those containing the eighth nerve nuclei. None showed degenerative changes similar to those described. The eighth cranial nerves in the 2 cases studied were normal.

Considered as a whole, the findings suggest that streptomycin can cause a specific destructive effect on the neurones of the eighth cranial nerve nuclei, especially the ventral cochlear nuclei (upon which discriminative hearing depends) and possibly the inferior vestibular nuclei. These changes would seem to be sufficient in some of the clinical cases to account for deafness and perhaps for vestibular dysfunction.

15875

Effects of Digitalis on Electrolytes of Heart Muscle.*

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The basic mechanism whereby digitalis exerts its specific actions on the heart is not fully known. The changes effected by digitalis are evidenced in the electrocardiogram. These are electrical phenomena which suggest quantitative and distributive changes in the electrolyte pattern of heart muscle. Since it is impossible to determine the electrolyte change as effected by digitalis for one heart beat, it was thought that the total change effected by digitalization could be used as an index of the changes of an individual cycle. It was therefore considered worth while to make a quantitative study of the electrolytes of both auricular and ventricular muscle after maximal digitalization.

Experimental Method. Fifty dogs, anesthetized with pentobarbital sodium, were divided into 3 groups. Nineteen animals were used to determine the normal distribution of electrolytes in both auricular and ventricular muscle. Twenty-one animals received tincture of digitalis intravenously (0.1 cc per kg of body weight) at 5-minute intervals until death. They required an average of 11 doses (range 9-13). To rule out any effect of the alcohol in the tincture of digitalis, 10 additional dogs were given, in an identical manner, the equivalent amount of alcohol present in a lethal dose of the tincture.

Tissue from each of the 4 chambers was analyzed separately. Chlorides and total nitrogen were determined on fresh tissue. The remainder was dried to constant weight for water determination. One- or 2-g samples of the dried pulverized tissue were ashed at 550°C for 16 hours, and the ash dissolved in 0.5 N HCl (25 or 50 cc respectively). Ca, Fe, Mg, Total P, K and Na were determined from the solution of ash (see ref. 1, 5, 7-11 for methods).

Results. The results of the control group are summarized in Table I, those for the experimental group in Table II, and those for the alcohol group in Table III. Table IV summarizes the changes which were statistically significant.

Discussion. While the water content of both auricular and ventricular muscle was comparable in both the experimental and the control groups, the electrolyte content of ventricular muscle in all 3 groups was much greater than that of auricular muscle. An absolute change in sodium, potassium, and calcium content occurred. There was a significant decrease in potassium and calcium in auricular muscle and a gain in sodium. The gain in sodium did not compensate for the

TABLE I.
Control (19 Animals).
Electrolytes in mEq. per 1000 g of Dry Tissue.

	R.A.	L.A.	R.V.	L.V.
Calcium	16.6	16.3	11.5	11.2
Chloride	77.8	81.6	87.3	87.8
Iron	7.6	7.6	11.6	12.1
Magnesium	44.4	45.3	75.6	79.0
Phosphorus	180.0	185.0	254.0	261.0
Potassium	137.0	154.0	277.0	279.0
Sodium	96.6	121.0	253.0	255.5
Total Nitrogen %	2.36	2.48	2.90	2.96
Water %	74.6	74.9	77.1	77.4

* Taken from a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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TABLE II.
Electrolytes in mEq. per 1000 g of Dry Tissue.

	R.A.	L.A.	R.V.	L.V.
Calcium	12.6	12.7	9.0	9.1
Chloride	83.4	86.8	91.5	93.0
Iron	7.2	7.4	12.6	13.8
Magnesium	41.1	43.6	74.8	79.7
Phosphorus	169.0	185.0	248.0	257.0
Potassium	111.0	121.5	268.6	270.0
Sodium	152.2	165.8	306.0	326.0
Total Nitrogen %	2.38	2.53	2.91	2.97
Water %	74.3	75.2	76.9	77.5

TABLE III.
70% Alcohol (10 Animals).
Electrolytes in mEq. per 1000 g of Dry Tissue.

	R.A.	L.A.	R.V.	L.V.
Calcium	16.2	15.9	11.3	11.5
Chloride	80.0	83.4	86.5	87.6
Potassium	141.5	156.6	277.8	277.7
Sodium	102.5	118.6	249.0	260.5
Water %	75.2	75.4	76.7	76.6

loss of potassium and calcium (Table IV). Ventricular muscle showed an absolute increase in sodium in the absence of any other significant changes. The loss of potassium from auricular muscle may be explained on the basis of the vagal component of the action of digitalis, since vagal stimulation causes an outflow of potassium from auricular muscle.[2,3] The mechanism of an absolute increase in sodium in heart muscle is not understood, but may be related to the poisonous actions of digitalis since an increase in sodium above the physiological ratio of this ion to potassium and calcium produces toxic effects upon the heart.[4,6]

TABLE IV.
Total Change in Electrolytes.*
(Milli-equivalents per 100 g of dry tissue.)

	R.A.	L.A.	R.V.	L.V.
Control Potassium	137.0	154.0	277.0	279.0
Experimental Potassium	111.0	121.5	268.6	270.0
Loss of Potassium	26.0	33.5	8.4	9.0
Control Sodium	96.6	121.0	253.0	255.5
Experimental Sodium	152.2	156.8	306.0	326.0
Gain in Sodium	55.6	35.8	53.0	70.5
Control Calcium	16.6	16.3	11.5	11.2
Experimental Calcium	12.6	12.7	9.0	9.1
Loss of Calcium	4.0	3.6	2.5	2.1

* From a statistical analysis of the data, only potassium, sodium, and calcium showed P-values not greater than 0.02.

Summary. 1. Maximal digitalization caused a decrease in the potassium and calcium content of auricular muscle and a gain in sodium which does not appear to compensate for the loss of potassium and calcium. Ventricular muscle showed a gain in sodium which does not appear to compensate for the loss of potassium and calcium. Ventricular muscle showed a gain in sodium in the absence of any other significant changes. 2. The changes in sodium, potassium and calcium following digitalization occurred in the absence of significant changes in the water content of the digitalized heart.

15876

Effects of Pyribenzamine and Benadryl on Chick Embryo and on Vascular Phenomenon Induced by Normal Serum.

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During the last few years extensive studies have been carried out on the toxic, antihistaminic and antianaphylactic properties of pyribenzamine, benadryl and related compounds. Data on the effects of antihistaminic drugs on the chick embryo and upon the inverted anaphylactic shock induced by Forssman antibodies are presented in this communication.

Material and Methods. Fertilized chicken

eggs were incubated at 42°C for 3 days. The eggs were removed from the shell, placed in a glass beaker (150 ml), and kept at 37°C. The vascular phenomenon was induced by the addition of normal rabbit serum, as described by Witebsky and associates.[1,2]

Pyribenzamine hydrochloride (N′pyridil-N′benzyl-N-dimethylethylenediamine HCl) was supplied by Ciba Pharmaceutical Products, Inc. through the courtesy of Dr. F. L. Mohr; benadryl hydrochloride (β-dimethylaminoethyl benzohydryl ether HCl) by Mr. F. H. Nelden of Parke, Davis and Co.; hetramine (N,N-dimethyl-N′benzyl-N′ [α-pyrimidyl] ethylene-diamine) by Dr. John V. Scudi of the Pyridium Corp. The drugs were dissolved in buffer solution (pH 7.4) prepared with Na_2HPO_4 and KH_2PO_4.

Experimental. A representative experiment illustrating the toxic effects of pyribenzamine and benadryl upon the embryonated chick ensues. Decreasing amounts of the drugs (volume 1 ml) were slowly dropped on the chick embryo; 4 eggs were used for each concentration. Both pyribenzamine and benadryl in amounts of 2.5 mg caused almost immediate bradycardia, followed within 2 minutes by stoppage of the heart beat and death of the embryo. In some instances, hemorrhages appeared in the embryo or in the vascular network. In smaller amounts (1.25 mg and 1 mg) both compounds caused bradycardia and apparent stoppage of the heart beat. Often, complete recovery took place after a lapse of several hours. Hemorrhages were noted only occasionally. Addition of the drugs in amounts of 0.5 mg and 1 mg resulted in bradycardia and only rarely in transitory stoppage of the heart beat. In yet smaller amounts (0.1 mg to 0.0001 mg) the compounds did not cause any visible changes. Hetramine,[3] another antihistaminic compound, too, caused transitory bradycardia or stoppage of the heart beat, depending upon the amounts used.

The vascular phenomenon, which was studied in detail by Witebsky and co-workers,[1,2] is due to the reaction between the Forssman antigen of the chick embryo and the corresponding antibodies. It resembles closely the inverted anaphylactic shock. In view of the relationship between true anaphylaxis and histamine poisoning, it seemed of interest to determine whether or not antihistaminic compounds affect the inverted anaphylactic shock of the embryonated chick. However, since histamine even in large amounts (up to 50 mg of histamine diphosphate) has no effect upon the 3-day-old chick embryo, it can be postulated that the antihistaminic compounds do not prevent the vascular phenomenon. That this is actually the case has been shown in numerous experiments, in which nontoxic doses of pyribenzamine and benadryl, administered prior to or concomitant with Forssman antibodies (normal rabbit serum), failed to prevent the vascular phenomenon.

Discussion and Summary. The experiments reported here revealed that the antihistaminic compounds pyribenzamine, benadryl and hetramine in the 3-day-old chick embryo cause bradycardia, stoppage of the heart beat and occasionally hemorrhages. It appears likely that the chick embryo can be used to advantage in studies on the relative toxicity of various antihistaminic compounds. The drugs do not prevent the inverted anaphylactic shock, which is due to factors other than histamine.

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15877

Attachment of Sedentary Marine Organisms to Petrolatum Surfaces.

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Independent observations, in our laboratories, in the Clapp Laboratories, and in the laboratories of the International Nickel Company, and of the Mellon Institute,† have shown that sedentary marine organisms attach much less readily to glass, metal or plastic surfaces coated with petrolatum than to similar uncoated surfaces. The present paper presents the results of an analysis of the rationale of this effect, and certain relevant data on the antifouling action of other compounds. The antifouling effect of petrolatum might be attributed to its unsubstantial character, its hydrophobic nature, or some specific repellant or toxic characteristic. We have compared petrolatum mixtures of varying consistency, and mixtures containing wetting agents to modify the hydrophobic character; we have found no clear correlation with either property.

Methods. Preliminary tests with the various materials were carried out in the laboratory. Briefly, it was found that surfaces which permitted successful attachment of sea anemones by their bases, or of mussels by their byssus pads, or which permitted successful attachment or locomotion by asteroids (sea stars) or gastropods (tectibranchs), were unsuitable for preventing attachment of sedentary organisms in general. On the other hand, surfaces which prevented attachment of the test animals *(Pisaster ochraceus, Tethys californicus, Cribrina xanthogrammica, Mytilus californianus)* in the laboratory were often effective antifoulants.

Actual exposure tests were conducted with glass or metal plates, coated with the material to be tested, and suspended either in the open sea at La Jolla or in the entrance channel to Newport Harbor at Corona del Mar. The sites and biological characteristics of the sedentary populations have been described by Coe,[1] Coe and Allen,[2] and Scheer.[3] The panels were examined at regular intervals, qualitatively at La Jolla, and semiquantitatively at Corona del Mar. Photographic records were kept of plates exposed at La Jolla.

Results. Table I gives the results of several tests with petrolatum (petrolatum white U.S.P.) In every case, coated panels were less heavily fouled than control uncoated surfaces. Two series of mixtures were then tested, to study the effects of varying consistency. These mixtures were made by diluting a crude petrolatum with lubricating oil, or with crude naphthenic acids from petroleum. The A.S.T.M. penetration values for these mixtures ranged from 93 to 450 at 77°. No consistent differences were observed in antifouling properties. However, purified white petrolatum was more effective in retarding attachment than were crude mixtures of comparable consistency.

Oil-soluble wetting agents of many different types were incorporated in petrolatum, to alter its hydrophobic properties. In an exposure at Corona del Mar, there was no relation between wettability of surface, as measured by contact angle with sea water (Adam[4]), and extent of fouling (Table II). One compound, a sodium alkyl sulfonate of uncertain composition, was markedly superior to all others tested in preventing attachment of organisms, although it was not the most effective wetting agent. Similar, though less

* Present address: School of Medicine, University of Southern California.

† Personal communication from Dr. William F. Clapp.

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TABLE I.
Settlement of Organisms on Aluminum Panels (A) and on Aluminum Panels Coated with Petrolatum (P) Exposed at the Same Time. Corona del Mar.†

Duration of exposure, wks	Coating	Time for 10% coverage by ABH,* days	Avg % of area covered by Bryozoans	Maximum Bryozoans	No. Tubeworms
10	A	3	18		
	P	7	0		
28	A	2	30	3	19
	P	2	0	3	2
20	A	12	5.5	41	0
	P	3	0	1	0
24	A	6	30	39	1
	P	17	0	10	2
38	A	8	28	68	9
	P	11	0	36	18
28	A	3	47	62	14
	P	5	1.1	34	29
8	A	5	1.3	110	14
	P	10	0	1	0
14	A	2	47	129	92
	P	5	0	11	2

* ABH signifies the film of algae, bacteria, and hydroids characteristic at the early stages of fouling (Scheer[3]).

† The different panels were exposed at different seasons. This accounts for the variation of intensity of settlement.

TABLE II.
Antifouling Efficacy of Surface-active Materials Incorporated in Petrolatum. Exposed at Corona del Mar Beginning December 14, 1943, for 14 weeks.

Materials*	Chemical nature	Conc. in petrolatum %	Contact angle		ABH		
			Out	In	Time, wks, for coverage of 10%	50%	Avg coverage
Aerosol-OT	Dioctyl disodium sulfosuccinate	10	45°	45°	0.65	3.2	39
Armour's AM 1180	Octadecyl amine	2	45	55	0.50	2.5	45
" " 2180C	Octadecyl diamine and triamine	9	45	60	1.1	10.0	34
Emery's A 105 (R)	Aliphatic amine	10	45	60	0.52	2.6	48
" X 68 (R)	Aliphatic ester of polyglycol	20	45	50	2.0	8.8	29
Saponin	Phenanthrene glycoside	22	45	75	2.9	11.0	23
"	" "	9	45	90	4.0	10.0	21
Aerosol-OT		4	60	65	1.1	11.0	29
Emery's X 72 (R)	Aliphatic ester of polyglycol	20	60	70	1.1	11.0	24
" T 333 (R)	Sodium alkyl sulfonate	20	70	70	11.0	11.0	4
Armour's AM 1120	Dodecyl amine	2	70	75	2.2	7.2	32
Emery's X 68 (R)		4	75	75	0.60	3.0	32
" " 72 (R)		4	80	90	1.8	11.0	22
Petrolatum			90	100	1.6	11.0	22

* The trade names serve to identify the materials used; their exact composition is unknown in several instances.

clear cut, results were obtained at La Jolla.

A second series of tests, using a series of sorbitol derivatives, confirmed the lack of correlation with wettability. The results of this series are given in Table III. The time required for development of a film of algae, bacteria, and hydroids (A B H) varied considerably among different compounds, as did the intensity of coverage and the number of settlers. This variation was in no way related to the surface action of the compounds; we may note, for example, the heavy settlement on the sorbitol ether, and on the modified hexastearate, which differed markedly in their surface action.

The results obtained in these tests suggest

TABLE III.

Antifouling Efficacy of Sorbitol Derivatives. Exposed at Corona del Mar Beginning July 14, 1944, for 8 Weeks. (All compounds dissolved in petrolatum to make a 10% mixture.)

Substance	Contact angle		Time, wks, for ABH coverage of		Avg coverage, %		Maximum No.	
	Out	In	10%	50%	ABH	Bryozoans	Bryozoans	Tubeworms
Sorbitan hexaoleate, modified	35°	50°	2.2	8+	12	0	3	1
Sorbitol ether	35	60	1.8	2.7	61	11	43	112
Sorbitan distearate	60	75	0.5	8+	33	8	18	12
Sorbitan oleate, modified	70	85	2.0	8+	27	0	23	0
Sorbitan monooleate	70	85	2.0	8+	20	0	6	0
Sorbitan trioleate	80	90	4.0	8+	15	0	11	0
Sorbitan triricinoleate	80	95	1.5	4.9	52	0	34	2
Sorbitan hexastearate, modified	85	95	0.4	2.0	40	26	80	34
Sorbitan tristearate	90	95	0.4	1.9	52	2.7	51	8
Petrolatum	90	100	0.7	8+	39	0	11	0

that the antifouling action of petrolatum might better be attributed to specific chemical properties of unknown nature than to either consistency or hydrophobic properties. Certainly various modifications in chemical composition produced more startling changes in antifouling properties than did alterations in consistency or wettability *per se*.

One such chemical change appeared to be worthy of further examination. The most effective petrolatum mixtures, in the prevention of attachment, were those containing a sodium alkyl sulfonate. We accordingly tested several other compounds of a similar nature.‡ The aliphatic sulfonates were all very effective as antifoulants when incorporated in petrolatum, while the sulfated ester (Aerosol OT) was somewhat less effective; sodium hydroabietyl sulfonate was decidedly inferior.

A possible explanation of the effectiveness of the aliphatic sulfonates might be the following: The sulfonic acid radical is polar and strongly acidic. Accordingly, at an oil-water interface, we would expect orientation such that the sulfonate radical will lie in the aqueous phase and will be ionized. This ionization will be accentuated if the solution is buffered to an alkaline pH, as is the case with sea water. This will provide, in effect, an acidic or negatively charged oil surface.

We were able to show that such orientation does occur by immersing panels coated with petrolatum-sulfonate mixtures in sea water, and measuring the change in wettability (contact angle) with time. With concentrations of sulfonate from 5 to 40%, wettability increased during the first 4 hours of immersion, rapidly at first and then more slowly. No further increase occurred during 20 hours of immersion. The importance of this orientation in prevention of fouling was shown in an experiment in which sulfonated material was mixed with a black asphalt paint. One panel was treated with this mixture and allowed to dry before immersion in sea water; a second panel was immersed in sea water for 2 hours immediately after preparation, and then allowed to dry. The second panel was distinctly superior in antifouling efficacy to the first (Table IV).

Summary. The action of petrolatum in delaying or preventing the attachment of sedentary marine organisms to submerged surfaces is not consistently altered by changes in consistency, produced by dilution with miscible liquids, or in wettability, produced by incorporation of wetting agents. The alterations observed appear rather to be the result of unknown chemical differences in the coatings. One such difference, characteristic of mixtures of petrolatum with aliphatic sulfonates, was shown to be related to orientation at the surface. It is suggested that the

‡ Aerosol OT (dioctyl disodium sulfosuccinate); T-333 (R), T-284 (R), T-285 (R), T-286 (R) (aliphatic sulfonates, Emery Industries, Inc.); Gamonol X, Sulfoline P (sulfonated petroleum products); Sodium hydroabietyl sulfonate (Hercules Powder Co.).

TABLE IV.
Effect of Immersion in Sea Water on Antifouling Efficacy of a Paint-Sulfonate Mixture. Panels exposed at Corona del Mar for six weeks beginning October 17, 1944.

Material	Time for 10% ABH coverage, days	Avg coverage		Maximum No.		
		ABH	Bryozoans	Bryozoans	Tubeworms	Barnacles
Black asphalt paint	6	24	0	44	6	16
Same + 30% T-333	5	37	0	0	0	0
Same + 30% T-333, soaked 2 hr in sea water	14	23	0	1	0	0
Same + 50% T-333, soaked 2 hr	42+	5	0	0	0	0

interface between such a mixture and sea water has a negative charge, and that this may be important in its antifouling properties.

The authors are indebted to Messrs. Harold Schiller and Don Bowers of the General Petroleum Company of California for helpful suggestions and for assistance in the preparation of certain materials. Materials were also supplied by the North American Aviation Co., Emery Industries, Inc., the Atlas Powder Co., the Hercules Powder Co., the Monsanto Chemical Co., and Armour and Co. The technical assistance of Miss Margaret L. Campbell was of the greatest value in a major part of the work at Corona del Mar. Helpful suggestions were made by Dr. A. C. Redfield, of the Woods Hole Oceanographic Institution.

15878 P

Effect of Drugs on Gastric Motility Following Vagotomy.

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(Introduced by Keith S. Grimson.)
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During the past 4 years, reports by Dragstedt,[1] Grimson,[2] Moore[3] and their associates have renewed interest in vagotomy for the management of patients with peptic ulcer. Although prompt relief from symptoms and healing of ulcer usually follow vagus resection, adverse complaints may develop because of reduced gastric peristalsis with retention or delayed emptying of stomach contents. Investigation of this retention phenomenon led to studies of gastric motility and tone as shown by variations of intragastric pressure in rabbits and in patients before and after vagotomy and after drugs.

Methods. Experiments were performed on rabbits after bilateral cervical vagotomy using local anesthesia and frequently inserting tracheotomy tubes. Intragastric pressures were recorded graphically by water manometers connected through small catheters to stomach balloons inflated with 30 to 50 cc of air. Drugs were administered hypodermically 30 minutes to 2 hours after vagotomy. Records of gastric motility and of resting intragastric pressure or "tone" were made for periods of 2 hours or longer. Experiments were also performed in rabbits one day to 8 months after transthoracic vagotomy done through a left thoracotomy wound. Studies on patients were obtained before and at intervals after transthoracic vagotomy for peptic ulcer. The balloons used in patients were inflated by 300 cc of air and pressures

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TABLE I.
Effects of Drugs in Rabbits.

Drug	No. animals	No. injections	Contractions	Tone
Pilocarpine	4	4	+++	+++
Physostigmine	4	5	±	0
Pituitrin	4	4	±	+
Priscol	4	8	0	+
Histamine	4	6	+	+
Neostigmine	4	7	+	++
Mecholyl	6	14	+	++
Doryl	9	17	+++	++++
Urecholine	4	7	+	+++

\+ = 0 to 2 cm
++ = 2 to 3 cm
+++ = 3 to 4 cm
++++ = over 4 cm

TABLE II.
Effect of Drugs in Patients.

Drug	No. tests	Dose and route	Onset, min	Contractions	Tone
		Preoperative			
Neostigmine	6	0.5-1.5 mg subq.	—	0	0
Doryl	3	.15-.25 " "	—	0	0
Mecholyl	4	0.2 g orally	—	0	0
Urecholine	3	10 mg U.T.	10	+	0
		Postoperative			
Priscol	3	10-30 mg subq.	—	0	0
Histamine	3	0.8 " "	—	0	0
Neostigmine	19	0.5-2.0 " "	—	0	0
Mecholyl	2	10 " "	2-5	+	0
"	8	0.2 g orally	—	0	0
Doryl	4	2.0 mg U.T.	60	++	+
Urecholine	5	1.0-2.5 mg subq.	2-5	+++	++
"	15	10-50 mg U.T.	120	+	+

\+ = 0 to 2 cm
++ = 2 to 3 cm
+++ = 3 to 4 cm
++++ = over 4 cm
U.T. = Under Tongue.

were recorded by a bromoform manometer. Drugs employed in patients were administered hypodermically, swallowed, or allowed to dissolve under the tongue.

Results. After bilateral cervical vagotomy all animals died usually within 24 hours and had pulmonary edema and pneumonia.

During the first several hours after operation, however, cessation of fluctuations in intragastric pressure and slight depressions of the resting tone occurred. Drugs were administered to 43 animals during this period of quiescence and some effected restoration of motility (Table I). The choline derivatives and pilocarpine produced strongest contractions and greatest increase of tone. Thirty rabbits survived transthoracic vagotomy 4 days or longer. Cessation of fluctuations of intragastric pressure and decrease of tone occurred during the first 12 to 48 hours. Thereafter hypermotility developed spontaneously and persisted in animals followed 8 months. Contraction waves were persistent, rapid, and regular with an amplitude 4 to 6 times greater than that before vagotomy. These movements were not effective, since gastric dilatation and retention occurred and was seen later at abdominal exploration. Gastric ulcers developed in several of these rabbits.

In patients after vagotomy fluctuations of intragastric pressure were low or absent and tone little changed or increased. Histamine, 2 benzyl-4, 5 imadazoline hydrochloride (Priscol) and Neostigmine produced no change of tone or return or contractions (Table II). Acetyl-beta-methyl-choline chloride (Mecholyl) subcutaneously produced a slight return of contractions. Mecholyl bromide orally had no effect, and carbamylcholine

chloride (Doryl) orally resulted in a slight increase of tone with good contractions. Urethane of beta-methyl-choline chloride (Urecholine), injected subcutaneously, raised the tone baseline and produced excellent contractions lasting as long as 4 hours. Administered orally, Urecholine stimulated fair contractions. The effect appeared slowly, but was prolonged.

Conclusions. Death of rabbits one or 2 days after cervical vagotomy occurred as reported by Short[4] and others. During the first several hours after operation the animals seemed in good condition and stomach motility ceased. Levin[5] has described this as "shock." Motility recovered later and persisted as described by Opuls,[6] Auer[7] and others. Van Yzeren[8] noted chronic gastric ulcers in rabbits after vagotomy and this has been confirmed by others and by us.

In man gastric contractions were markedly depressed after vagotomy even though resting intragastric pressure increased slightly. These effects persisted. Ulcers healed or became quiescent. This difference in the effect of vagotomy, that is, healing of ulcer in man and development of ulcer in rabbits might be associated with decreased motility in man and increased or continued motility in rabbits.

Of the drugs studied (Table I) most restored motility or tone during the first hours after vagotomy in rabbits. In patients Urecholine was the most effective. Starr and Ferguson[9] first reported the clinical use of Urecholine. Machella *et al.*[10] described its effect in patients after vagotomy. Our observations confirm theirs but indicate also that some effect was obtained from Mecholyl and Doryl. Urecholine and Doryl have proved effective in overcoming gastric retention in patients treated by vagotomy providing scar tissue obstruction of the outlet of the stomach does not exist.

4 Short, R. H. D., *J. Path. and Bact.*, 1944, **56**, 355.

5 Levin, P. M., *J. Pharm. and Exp. Therap.*, 1938, **62**, 449.

6 Opuls, W., *J. Exp. Med.*, 1906, **8**, 181.

7 Auer, J., *Am. J. Physiol.*, 1909, **25**, 334.

8 Van Yzeren, *Z. f. klin. Med.*, 1901, **43**, 181.

9 Starr, I., and Ferguson, L. K., *Am. J. Med. Sc.*, 1940, **200**, 372.

10 Machella, T. E., Hodges, H. H., and Lorber, S. H., *Gastroenterology*, 1947, **8**, 36.

15879

Nutrition of Trout: Studies with Practical Diets.*

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Introduction. Both in anatomy and habit the trout appears to be a carnivore. In hatchery practice it was early established that these fish can live and grow to maturity on diets of animal origin.[1] Liver has been shown to be an adequate ration in itself and any modification of the ration has been related to attempts to replace liver with less expensive organs and animal products. Plant materials have been thought to have little promise. Embody[2] was unable to raise trout on a mixture of animal and plant materials. Subsequently he made proximate analyses of the stomach contents of fish raised under

* Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

1 McCay, C. M., Bing, F. C., and Dilley, W. S., *Trans. Am. Fish Soc.*, 1927, **57**, 240.

2 Embody, G. C., *Trans. Am. Fish Soc.*, 1918, **48**, 26.

3 Embody, G. C., and Gordon, M., *Trans. Am. Fish Soc.*, 1924, **54**, 185.

natural conditions,[3] and found carbohydrates to be low, protein, mineral and fat high, and the presence of considerable chitinaceous material.

McCay[1,4] utilizing purified rations of the casein-sucrose type, indicated that trout require fresh liver. Titcomb *et al.*[5] raised trout on mixtures of liver with fish and vegetable meals. More recently, Wilkinson,[6] in an extensive study on rainbow, brook, and brown trout found that mixtures similar to Titcomb's produced trout more economically than those completely of animal origin.

Field *et al.*[7] reported that vegetable meals supplemented with skim milk powder, gelatin, and concentrated liver fractions produced fair growth for a short period. Hatchery experiments[8] with one of these combinations showed that after 8 weeks growth ceased, but was resumed again when the ration was finely ground. Laboratory trials indicated that skim milk powder was an essential ingredient in this mixture, while liver fractions contributed little to the growth of the fish on these rations. The following laboratory and hatchery experiments were set up to improve Field's ration.

Experimental. All the laboratory experiments were conducted in the biological laboratories of the James Nevin State Hatchery, Madison. Healthy yearling rainbow trout *(Salmo gairdnerii irideus)* were obtained directly from the stock raceways of the hatchery. Fish weighing 18-20 g were selected and divided into groups of 25. The average weight of fish was obtained weekly using the method outlined by Field *et al.*[7] Hemoglobin determinations were made at intervals on blood obtained by heart puncture. The hemoglobin was measured as acid hematin. At the termination of the experiment representative fish from each group were killed, weighed, and the livers removed and weighed. A control group of fish received a ration made up of liver, canned carp, and yeast, the stock diet in use at the Nevin State Hatchery. Other groups received Ration C and various modifications thereof. Ration C, Field's basal ration,[7] modified to increase the protein content and reduce the cost, consisted of: skim milk powder 25, alfalfa leaf meal 10, soybean oil meal 15, liver A† 10, brewer's yeast 5, iodized salt 0.5, red dog flour 22.5, cod liver oil 2, and gelatin 10 (either as capsules or finely ground). Vitamin C was added at the rate of 4.54 g/100 lbs of ration. Ration C (including the gelatin capsule, see below) is calculated to contain protein 40%, fat 5%, carbohydrate 45%, ash 5% and fiber 5%. The stock ration on the same basis contains protein 66%, fat 24%, carbohydrate 4%, ash 6%, and fiber 0%. The stock diet was fed by dropping an amount, approximately equal to 6% of the body weight, into the tank while Ration C and modifications were fed by gelatin capsule[9] at an isocaloric level (2.0% of the body weight). Results are recorded in Table I in terms of growth, hemoglobin, liver size and mortality. The liver to body weight ratio has been shown earlier[10] to be an important index of the health of the fish. Enlarged, pathological livers are associated with high mortality.

From the data presented in Table I it is evident that sprouted soybeans are inferior to the ordinary soybean oil meal (expeller process). After 7 weeks on Ration D, soybean sprouts, autoclaved at 10 lbs pressure for 10 minutes, were substituted and growth for the next 9 weeks was somewhat improved; but the enlarged livers produced by the raw-sprouted beans were not reduced in size. When soybean oil meal was replaced by increasing the red dog flour to 37.5%, no sig-

[4] McCay, C. M., and Dilley, W. E., *Trans. Am. Fish Soc.*, 1927, **57**, 250.

[5] Titcomb, J. W., Cobb, E. W., Crowell, M.F., and McCay, C. M., *Trans. Am. Fish Soc.*, 1928, **58**, 205.

[6] Wilkinson, J. T., *Trans. Am. Fish Soc.*, 1938, **68**, 96.

[7] Field, J. B., Herman, E. F., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 227.

[8] Field, J. B., unpublished data.

† Liver A is a product of Wilson and Company, Chicago, Ill.

[9] Field, J. B., Herman, E. F., and Elvehjem, C. A., *Copeia*, 1944, No. 3, 184.

[10] McLaren, B. A., Herman, E. F., and Elvehjem, C. A., *Arch. Biochem.*, 1946, **10**, 433.

TABLE I.
Results of Laboratory Feeding Trials with Different Vegetable Meals and Levels of Yeast.

Ration	Modification	Weekly gain (16 wks, g)	Hemoglobin g/100 ml blood	Liver wt/body wt × 100	Mortality, No. of fish
Series I					
Stock	Liver—canned carp	2.4	8.6	1.1	1
C	Field7 as modified in text	2.3	8.2	1.1	1
D	Sprouted soybeans replacing soybean oil meal	0.7 (7 wks)	7.1	3.1	1
	" " autoclaved 10 lbs for 10 min	1.6 (9 wks)	7.0	3.0	1
E	Red Dog Flour replacing soybean	2.1	7.6	1.4	2
F	Ground oats replacing Red Dog Flour	2.5	8.4	1.0	1
G	8% wheat germ flour replacing 8% Red Dog Flour	2.3	6.9	1.2	1
H-1	Brewer's yeast omitted—poultry yeast 5%	1.7	4.1	4.0	2
2	" " " " 10%	1.5	4.2	6.5	9
3	" " " " 15%	1.6	3.5	7.2	12
Series II					
C		1.6	10.5	1.4	0
N	Glandular meal replacing liver A	1.7	12.5	1.5	0
M	Flavonne meal replacing skim milk powder	0.6	6.8	1.8	1

nificant changes were noted. When Ration C was modified by replacing the red dog flour by finely ground rolled oats, growth, hemoglobin and liver to body weight ratio were the best of any group in the series. Substitution of 8% of the red dog flour in Ration C by wheat germ flour, a good source of vitamins, and minerals, lowered the hemoglobin from 8.2 to 6.9 and did not improve growth. When the brewer's yeast (Anheuser Busch) was omitted and 5% of livestock and poultry grade yeast substituted (Ration H-1) growth was poor, hemoglobin low, and the livers enlarged, lobulated and mustard yellow in color. Higher levels of this yeast augmented these deleterious effects and increased mortality.

As the foregoing experiments were being concluded 2 commercial products were brought to our attention, glandular meal and flavonne meal. The producers suggested that they could be substituted for liver A and skim milk powder respectively. The effect of these substitutions in Ration C is presented in the second series in Table I. In comparison with the controls it is apparent that glandular meal is equal or superior to liver A and at present is considerably cheaper. Flavonne meal cannot be considered as a substitute for skim milk powder.

In the second series the control group receiving Ration C did not grow as well as in the preceding experiment. The selection of fish of standard size (18-20 g) from the same hatch, now 4 months older, probably allowed the use of the stunted, slower growing fish.

It seems apparent from these experiments that under laboratory conditions it is possible to force feed yearling trout a practical ration and produce good growth, satisfactory hemoglobin values, and livers of normal size, shape and color. Using these factors as criteria of health, then the rations C, F, and G compare favorably with the fresh liver-canned carp control diet. These rations contain no fresh meat and are composed of 55% plant materials.

The question arises whether fish would eat such a ration under hatchery conditions. To test this, field experiments were devised and

TABLE II.
Results of Large Scale Feeding Trials at Westfield Hatchery by Mr. E. W. Hackman.

	No. of fish	Weekly gain 11 wks	Mortality No. of fish 6 wks	11 wks
Ration C	1400	0.86	19	38
Pork spleen	700	0.91*	313	—
Pork spleen plus yeast	700	0.77	5	99

* Weekly gain for 5 weeks. The figure reflects the loss of all the weaker more slowly growing fish from furunculosis.

carried out in the Westfield State Hatchery under the direction of Mr. E. W. Hackman. Ration C was selected for the field trial since it gave almost as good growth as Ration F, and was more economical.

Four groups of 700 each of 10 g brook trout *(Salvelinus fontinalis fontinalis)* were weighed and placed in separate tanks. The fish were hand-counted and hand-sorted to avoid injury. Two control groups received spleen, one with and one without poultry yeast. The remaining 2 groups were fed the experimental diets (Table II).

The ration was prepared in the dry form at the Department of Biochemistry and sent to the hatchery where it was mixed with 1½ times its weight of water and allowed to set (about 40 minutes) until it had a firm gelatinous consistency, not unlike liver. It was fed daily by dropping chunks into the tank.

Unfortunately 3 weeks after this experiment was set up, furunculosis broke out and spread throughout the hatchery. At the end of 6 weeks, 313 fish receiving spleen alone had died, and this group was discontinued. There was negligible mortality in the other 3 groups and these were maintained on their respective rations for an additional 5 weeks, until the disease had reached epidemic proportions. During this period 94 of the fish receiving spleen and yeast died and the rate of mortality increased rapidly during the last 2 weeks. Of the 1400 fish fed meatless rations only 38 died during the entire experiment.

Growth on Ration C was definitely better than on pork spleen even when supplemented with yeast. The 2 groups received slightly different liver preparations.

The weekly gain of these fish, while superior to that of the controls, was definitely lower than that of rainbow trout receiving Ration C in capsule (2.1 g/wk at 11 weeks). These fish were only half the weight of those fed in the laboratory experiments and would not be expected to make as great an absolute increment in weight, in a given period, as the larger fish. When the gain in weight is expressed in per cent of the initial weight it is seen that these fish grew at an even faster rate (98% in self-fed as against 67% in capsule-fed at 11 weeks).

During the spring of 1945, the supply of fresh meat suitable for fish feeding greatly decreased. The Wisconsin Conservation Department had Garver's Supply Company, Madison, prepare 3 tons of Ration H-1. It was well mixed and finely ground. This feed was distributed among several trout hatcheries and a controlled experiment was set up in which 7000 yearling rainbow trout were fed this ration. 3500 of the same age were fed the spleen-carp mixture. Both groups received the same amount of feed on the wet basis. The fish were weighed at the beginning and end of the experiment. Over a 6-month period both groups grew at approximately the same rate,—dry ration 412% gain—meat ration 400%. The losses were negligible, 0.3% (dry ration) and 0.6% (meat mixture).

Discussion. The demonstration in the laboratory experiments that rations containing over 40% carbohydrate could successfully support essentially normal growth and health in yearling rainbow trout provides an interesting contrast to the earlier work with purified rations[10] where carbohydrates in excess of 20% (glucose, sucrose, lactose or raw

starch) produced marked liver pathology. Tunison *et al.*,[11] who fed these carbohydrates by capsule to fish receiving meat diets, also found that enlarged livers, rich in glycogen, resulted. The carbohydrate in rations C, E, F and G is largely in the form of starch and since the rations are not subjected to heat must be considered as raw. Two possibilities suggest themselves as explanations: (1) That a diet of natural foods contains some substance or substances which protect the fish against the deleterious effect of high carbohydrate. If this is the case these protective substances are apparently not, according to the work of Tunison,[11] present in animal organs. (2) That the physical nature of these natural materials with their intimate mixture of all the proximate principles exerts the protective effect observed.

Not all of the rations of natural materials prevented liver enlargement. Ration D, containing sprouted soybeans, and the H rations, in which poultry yeast replaced brewer's yeast, produced moderately to greatly enlarged livers. In the case of the H rations it cannot be easily argued that the substitution of one type of yeast for another resulted in the omission of a protective factor since enlarged livers were produced with Ration D (sprouted soybeans) in which there was no change in the yeast. Furthermore brewer's yeast does not give protection against excessive carbohydrate when included in purified rations.[10] It is possible in both Rations D and H that a toxic factor is involved which operates to produce liver enlargement independent of the carbohydrate level. At least in Ration D the factor is not destroyed by autoclaving. This would seem to indicate that it is not the trypsin inhibitor since the latter is heat labile.[12] At this stage of the investigation and without further work it is impossible to determine why these ingredients lead to liver enlargement.

The field trials at the Westfield Hatchery with Ration C and at Nevin Hatchery with Ration H indicate that essentially similar growth can be obtained when these rations are self-fed as when force-fed.

Summary. A practical meatless ration for trout containing 55% plant material is described which is essentially equivalent to a standard liver, canned carp diet so far as growth and hemoglobin levels are concerned. The effect of various substitutes in the ration is described.

Although this ration contains 45% carbohydrate, liver enlargement was not noted except when sprouted soybeans or poor quality yeast were included. Large scale field trials indicated that Ration C was superior to pork spleen, and equivalent to pork spleen supplemented with canned carp, as a diet for yearling brook trout on the basis of both growth and mortality.

We wish to thank Wilson Laboratories, Chicago, for the liver fractions; Armour and Company, Chicago, for the glandular meal; Dawes Vitamilk Company, Chicago, for the Flavonne.

Grateful acknowledgment is due D. Mott Cannon, M.D., for his helpful suggestions and interest and Mr. Wendell Anderson, foreman, Nevin State Fish Hatchery, for his wholehearted cooperation in supplying suitable fish for the laboratory experiments.

[11] Tunison, A. V., Brockway, D. R., Maxwell, J. M., Dorr, A. L., and McCay, C. M., N. Y. Conservation Dept., Cortland Rept. No. 11, 1942.

[12] Hamm, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1945, **161**, 635.

15880

Production of Acute Rheumatic-like Heart Lesions in Mice.*

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Klinge[1] and others[2,3] have reported that rabbits subjected to sublethal anaphylactic shock develop cardiac lesions resembling those seen in human rheumatic fever. More complete and convincing evidence to that effect was submitted more recently by Rich.[4–6] These investigations support strongly the thesis that rheumatic fever, as well as certain diseases related to it, is a reaction to parenteral contact with foreign protein to which the tissues have been sensitized. In this laboratory we have recently been engaged in studies of protein hypersensitivity in mice and it was suggested by one of us (Hall) that their hearts be examined histologically for rheumatic-like lesions. The present report is concerned with the production of acute cardiac lesions resulting from 4 parenteral injections of foreign protein.

Methods. The whites were separated from fresh hen eggs, diluted with an equal volume of distilled water, brought to a pH of 7.0 to 7.2, mixed to disrupt the small mucinous sacs, filtered through cloth, and diluted as necessary with distilled water. Refiltration was often required to remove the slowly precipitating mucins of slight solubility. Dilutions of 1 to 8 or higher were used intravenously and 1 to 2 intraperitoneally. All injections were made in volumes of 1 ml. Solutions were used within 2 to 6 hours after preparation, being refrigerated during that time and warmed to approximate body temperature just before injection. Mice of the Olitsky, "typhoid resistant" strain were raised in the laboratory.

Several experiments were conducted using 3 intraperitoneal injections, given at 2-day intervals, followed by a 4th either intraperitoneally or intravenously on the 4th, 7th, 10th, 14th, or 21st day after the 3rd dose. Survivors were held for 7 days, sacrificed, and the tissues fixed in formalin. Serial sections of the heart were cut at 6 μ and stained with phosphotungstic acid, hematoxylin and triosin. Another experiment was conducted using one intraperitoneal dose followed by 3 intravenous injections (Experiment 30 C) given 8, 15, and 22 days after the first dose. Finally, a similar experiment was run where the first 3 doses were intraperitoneal and the 4th intravenous (Experiment 30 D). Mice which died or were moribund as a result of the 4th dose were immediately autopsied and their tissues handled as described above. In Experiments 30 C and D, no more than one hour elapsed between the 4th dose and autopsy. Illustrations shown below were taken from 5 of the mice in Experiments 30 C and D. Mice were 6 to 8 weeks old when autopsied. Tissues from untreated mice of similar age were examined.

Results. Lesions resembling many of those seen in acute rheumatic carditis in man and those described by Rich in rabbits were observed in all experimental animals. The untreated mice, which may be assumed to be spontaneously exposed from time to time to various foreign proteins, had minimal findings which none the less might be looked upon as "rheumatic stigmata:" they showed

* Aided by the Mayer Fund of the Department of Experimental Medicine.

1 Klinge, F., *Ergeb. d. allg. Path.*, 1933, **27**, 1.

2 Vaubel, E., *Ziegler's Beitr.*, 1932, **89**, 374.

3 Hall, E. M., and Anderson, L., *Am. Heart J.*, 1943, **25**, 64.

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5 Rich, A. R., and Gregory, J. E., *Ibid.*, 1944, **75**, 115.

6 Rich, A. R., *Proc. Inst. Med. Chicago*, 1945, **15**, 270.

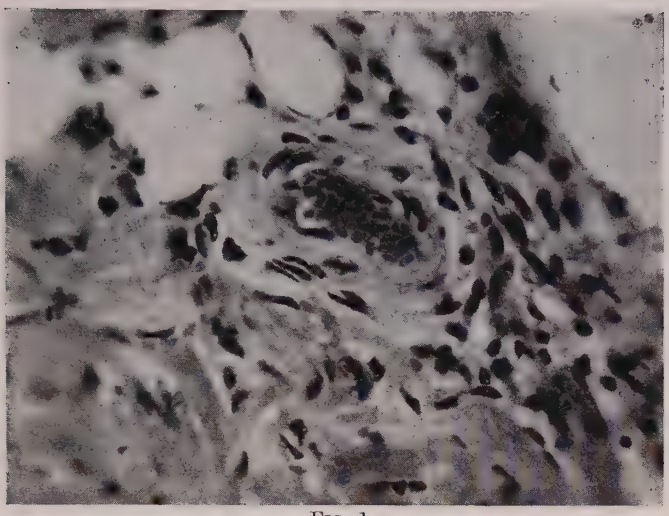

FIG. 1.
Sub-epicardial arteriole.

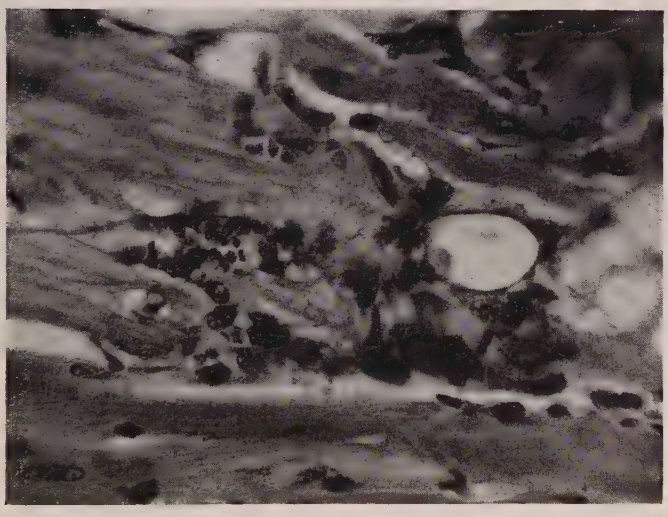

FIG. 2.
Hydropic degeneration of myocardial fibrils.

small and scattered areas of minor collagenous degeneration of the smaller coronaries, inconspicuous perivascular infiltrations, and moderate numbers of Anitschkow myocytes in those areas of greatest mechanical strain (*e.g.*, in the intervalvular septum).

The experimental hearts exhibited innumerable highly focal areas of acute vascular and perivascular degeneration of the reticular and collagenous connective tissue, with essentially complete fibrinoid degeneration of the arteriolar wall, as illustrated in Fig. 1. Fig. 2 illustrates the hydropic degeneration and disruption of myocardial fibrils. Fig. 3 shows a perivascular cellular infiltration in the wall of the left atrium. A low magnification of a moderately large infiltration near the base of a papillary muscle is shown in Fig. 4, the cellular constituents and degenerated central area being shown in higher magnification in Fig. 5. A verrucous lesion on the superior surface of a mitral leaflet is shown in Fig. 6. Such verrucae were seen frequently. Fig. 7 shows an acute verrucous

FIG. 3.
Perivascular infiltration in wall of left atrium.

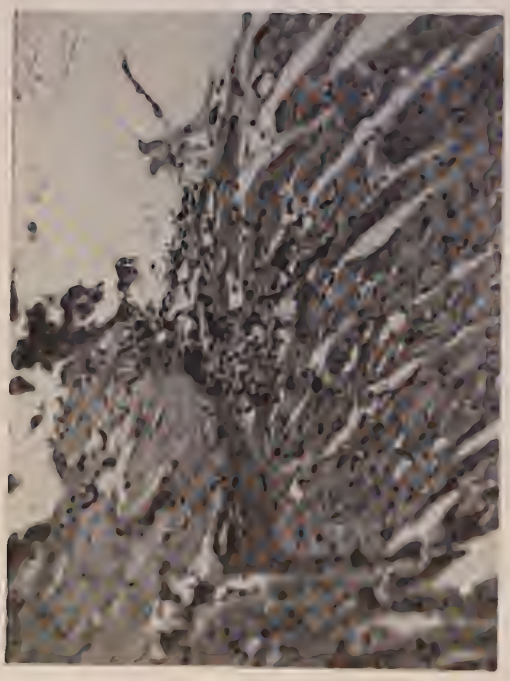

FIG. 4.
Infiltration near base of papillary muscle.

FIG. 5.
Cellular constituents in base of papillary muscle.

excrescence on the left septal wall just below the aortic valve. Fig. 8 shows the aorta and origin of the left coronary, with the septum and left ventricular wall and lumen below, and a high cut through 2 of the aortic cusps near their commissure. The pathologic fusion of these aortic cusps is seen in higher magnification in Fig. 9. A giant cell and degeneration of the collagenous and reticular tissue are evident, and while not apparent

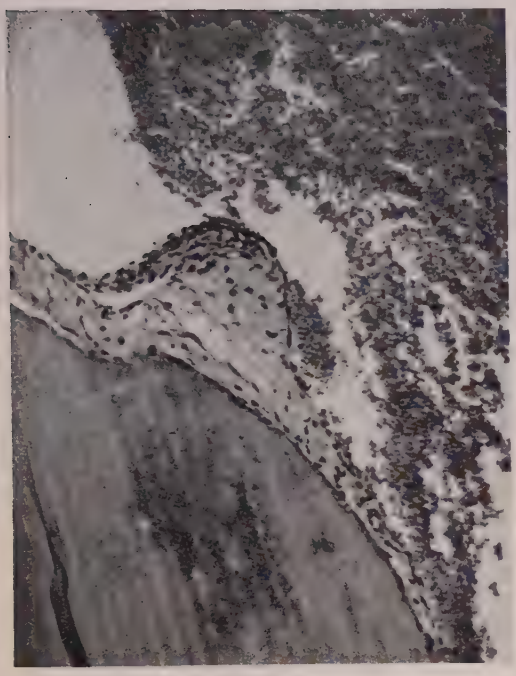

FIG. 6.
Verruca on superior surface of mitral leaflet.

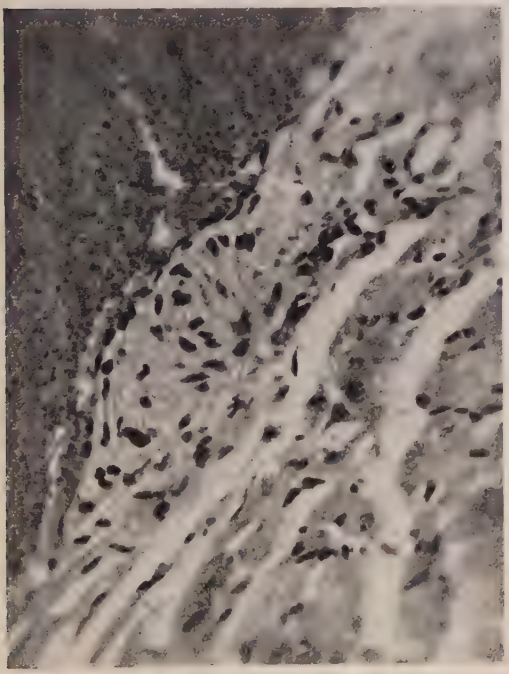

FIG. 7.
Verruca on left septal wall below aortic valve.

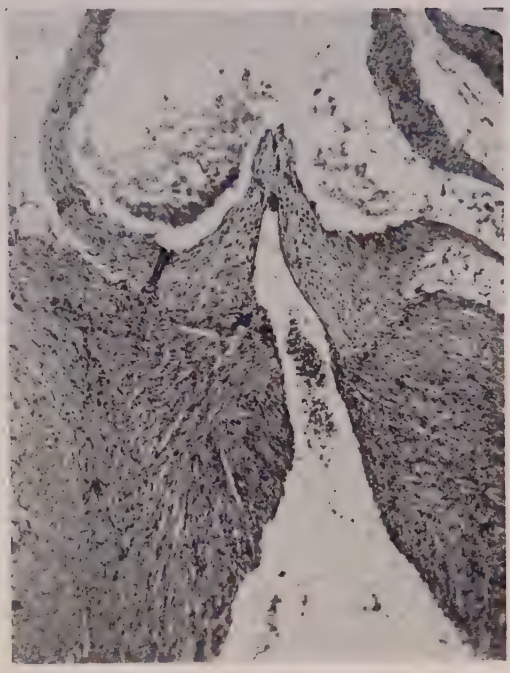

FIG. 8.
Fusion of aortic cusps near commissure.

FIG. 9.
Higher magnification of commissural fusion seen in Fig. 8.

in the reproduction, 4 other cells resembling giant cells were also seen at this point of fusion.

Discussion. The material presented above indicates that cardiac lesions may be produced in mice by parenteral readministration of foreign protein, and that they tend to simulate many of those reportedly found in rabbits under similar circumstances. They bear a striking resemblance to the lesions occurring in acute human rheumatic carditis.

It should be recalled that the mice illustrated in this report received only 4 doses of antigen in 22 days. Assuming, for lack of evidence, that the first injection was not injurious, and knowing that the animals were autopsied within one hour after the last dose, it would appear likely that most of the tissue changes must be ascribed to the 2nd and 3rd doses. The injury, therefore, could not easily have been of more than 2 weeks' duration in any animal. Furthermore, it was noted that animals receiving only a single "shocking" dose, and autopsied a week later, generally had much less florid lesions than those found in the hearts illustrated above. This suggests that repeated shocking doses may be more than additive and indicates that the preponderance of the lesions illustrated were not of more than one week's duration.

Further studies are in progress to define more fully the nature, variety, and incidence of these acute lesions and to investigate their production with other antigens. Studies are also in progress in which the mice are given opportunity to develop older, reparative lesions, and these with superimposed fresh ones. In addition, since it is theoretically impossible to obtain animals that have never been subjected to any foreign protein, the most careful investigation of untreated animals is desirable: the spontaneous occurrence of minimal rheumatic-like cardiac lesions in mice is too suggestive of human rheumatic fever to be overlooked or regarded merely as a small impediment to experimental work. Pathologic changes have also been observed in other organs of the mice reported here, and these will be separately described.

As has been pointed out recently by Weiser, Golub, and Hamre,[7] anaphylactic reactions in mice are slow to develop and prolonged in course by comparison with other animals. As noted by Perry and Darcy,[8] mice are relatively refractory to histamine, and unexpected relations between histamine and antihistamine drugs in mice have been reported by Mayer and Brousseau.[9] We know of no effort in mice or other animals, however, to induce cardiac lesions of the type described here with histamine. On the basis of current evidence, it is not entirely unlikely that these changes are not produced by the clinical anaphylactic reaction itself, for we have observed no relation between the severity of the clinical reaction at the time of protein injection and the severity of the pathological lesion resulting from it at a later date. For instance, mice which received all of their doses by the intraperitoneal route exhibited either no clinical reaction to the injection or had only the slightest and most questionable reaction. The cardiac lesions in these mice, however, were at least as advanced as in those inoculated repeatedly by the intravenous route and which developed acute and severe symptoms of cardiovascular and respiratory embarrassment with each succeeding dose. We are therefore tempted to conclude that some biochemical reaction occurs in the tissue which results in damage to them: if that primary reaction is induced by a sufficiently sudden access of antigen to the vascular tree, then frank clinical evidence of anaphylaxis may result, but if the antigen reaches the cardiovascular system more gradually, then there may be no clinical anaphylaxis but just as much fundamental tissue damage in the end.

While it is tempting to refer to the observed changes in mice as "rheumatic carditis," this would appear to remain unwarranted until it is shown first that the advanced lesions of myocardial scarring and valvular stenosis may be similarly produced; second that le-

[7] Weiser, R. S., Golub, O. J., and Hamre, D. M., *J. Inf. Dis.*, 1941, **68**, 97.

[8] Perry, S. M., and Darsie, M. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 453.

[9] Mayer, R. L., and Brousseau, D., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 187.

sions of other tissues correspond to those of human rheumatic fever and its related diseases; and third, some or most of the clinical and laboratory findings of the human disease are reproduced in animals. Until such time they might be called either "protein carditis," or "rheumatic-like carditis." If, however, one were to hypothecate that human rheumatic fever is the result of parenteral contact with foreign protein to which the tissues have been sensitized, one might expect that hypothesis to be reasonably compatible with the known relation of the streptococcus to the disease, for it would indeed be difficult to find a source better than the streptococcus of foreign protein which customarily, frequently, and intermittently gains access to the more intimate tissues and vascular system of man.

Summary. Acute rheumatic-like heart lesions have been produced in mice by parenteral injection of egg white on repeated occasions. Minimal lesions may also occur in untreated mice, possibly as a result of spontaneous sensitization and shocking by natural contact with protein. There was no apparent relation between the severity of clinical anaphylaxis and the severity of the pathologic changes in the heart.

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On Some Biological Characteristics of Streptomycin B.

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Fried and Titus[1] recently described the isolation, from streptomycin concentrates, of a new entity which they called streptomycin B. Chromatographic fractions described by these authors were made available for biological studies. The materials used for the present work were rich in streptomycin B and from Craig countercurrent distribution data[2] were estimated to contain not more than 10-15%, by weight, of streptomycins other than B. For purposes of comparison, studies with a highly purified preparation of streptomycin, free of streptomycin B, were included in the investigations. In this paper the term "streptomycin" will refer to the preparation containing no streptomycin B, while "streptomycin B" will refer to the above described chromatographic fractions rich in this latter material.

I. *In vitro studies.* The minimal inhibiting concentrations (M.I.C.) of streptomycin and streptomycin B for 5 strains or species of bacteria were determined in yeast beef broth, and for 2 species of mycobacteria in Kirchner's medium modified* to contain albumin and Tween 80 as in media described by Dubos and Davis.[3] The M.I.C. values thus determined are shown in Table I.

The importance of the type of culture medium used for such studies is clearly shown by the comparisons in Table II where addi-

1 Fried, J., and Titus, E., *J. Biol. Chem.*, 1947, **168**, 391.

2 Titus, E., and Fried, J., *J. Biol. Chem.*, 1947, **168**, 393.

* Modified Kirchner's synthetic medium for growth of *M. tuberculosis:*

Na_2HPO_4	3	g
KH_2PO_4	4	g
$MgSO_4$	0.6	g
Sodium citrate	2.5	g
Iron ammonium citrate	0.05	g
Asparagin	5.0	g
Glycerine	20	ml
Distilled water	1000	ml
Tween 80	0.05%	

Autoclave at 15 pounds for 20 minutes.

Solution of human serum albumin sterilized by filtration added to 0.1%.

3 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE I.
Comparative *in vitro* Activities of Streptomycin B and Streptomycin.

Test organism	M.I.C. in yeast beef broth*		Ratio Streptomycin B / Streptomycin
	Streptomycin-trihydrochloride γ/ml	Streptomycin B-hydrochloride† γ/ml	
Eberthella typhosa (Hobby)	9.94	12.2	1.23
Salmonella schottmulleri	8.42	13.5	1.61
Mycobacterium tuberculosis (H37Rv)*	1.01	3.73	3.70
Klebsiella pneumoniae (ATCC 9997)	1.53	5.70	3.73
*Mycobacterium smegmatis**	1.38	6.80	4.92
Staphylococcus aureus (Osgood)	1.50	7.80	5.20
Staphylococcus aureus (209-P)	1.35	9.55	7.06

* The two strains of mycobacteria were tested in a modified Kirchner's synthetic medium rather than in yeast beef broth.

† Several streptomycin B preparations all of approximately equivalent compositions were used for these studies. The figures given are averages of all data obtained.

TABLE II.
Effect of Culture Media on Relative Activities of Streptomycin and Streptomycin B Against Several Organisms.

Test organism	Material tested	M.I.C. in Yeast beef broth γ/ml	M.I.C. in Tryptone broth γ/ml	Ratio of M.I.C.'s Yeast beef/Tryptone	"Interference ratio"
K. pneumoniae	Streptomycin	1.53	0.0442	34.6	0.82
	Streptomycin B*	5.70	0.200	28.5	
	Streptomycin B/Streptomycin	3.73	4.53	—	—
E. typhosa (Hobby)	Streptomycin	9.94	0.188	52.8	1.15
	Streptomycin B*	12.2	0.200	61.0	
	Streptomycin B/Streptomycin	1.23	1.06	—	—
S. schottmulleri	Streptomycin	8.42	0.188	44.8	1.31
	Streptomycin B*	13.5	0.231	58.6	
	Streptomycin B/Streptomycin	1.61	1.23	—	—

* Several streptomycin B preparations all of approximately equivalent compositions were used for these studies. The figures given are averages of all data obtained.

tional data gathered in 0.75% tryptone broth, pH 8.5, are listed together with data taken from Table I. It perhaps should be emphasized at this point that the M.I.C. values listed in these tables are given in actual weight of material required to inhibit, and not in terms of streptomycin base. Since the streptomycin B preparations used were not entirely free of streptomycin, these figures will eventually have to be modified, and differences from streptomycin will undoubtedly be even greater than here shown.

It will be noted that in the various culture media used the M.I.C. values for streptomycin were always lower than those of streptomycin B. However, the manner in which a given culture medium affected the activity of either antibiotic depended on the test organism. Thus, examination of Table II reveals that for *K. pneumoniae,* 34.6 times as much streptomycin was required to inhibit growth in yeast beef broth as in 0.75% tryptone broth, pH 8.5. Of streptomycin B, 28.5 times as much antibiotic was required in yeast beef broth as in tryptone broth. The ratio $28.5/34.6 = 0.82$ we have called an

"interference ratio" and indicates that for this test organism yeast beef broth in comparison to tryptone broth interfered more with the action of streptomycin than with that of streptomycin B. On the other hand, the reverse was true when *E. typhosa* or *S. schottmülleri* were used as test organisms since the "interference ratios" for these 2 organisms were 1.18 and 1.31 respectively.

Since *K. pneumoniae* showed relatively greater sensitivity to streptomycin B as the culture medium was changed from a simple tryptone broth to a richer yeast beef broth, the possibility exists that the relative sensitivity of this organism towards streptomycin B *in vivo* might be even greater than *in vitro*. If this were so, one might expect *E. typhosa* and *S. schottmülleri* to show relatively less sensitivity to streptomycin B *in vivo*. Insufficient quantities of this material prevented testing it against all 3 organisms *in vivo*, but tests with *S. schottmülleri* in mice (see section on *in vivo* studies) lends evidence in favor of this hypothesis since the *in vitro* action of streptomycin was 1.2-1.6 times as great as that of streptomycin B for this organism (Table II) while *in vivo* the former was 3 times as active as the latter in experimental infection in mice.

It is of interest to note that at a recent conference on antibiotics, Hobby and Lenert[4] reported that certain residues, obtained in the preparation of crystalline salts of streptomycin, were from 2 to 5 times more active than highly purified streptomycin sulfate against a number of strains of *E. typhosa*. One of these strains was kindly supplied us by Dr. Hobby and its behavior towards streptomycin B was determined. The results of the tests with this organism are included in Tables I and II. It is to be assumed that Hobby and Lenert's[4] conclusions were drawn on the basis of activities given in streptomycin units as measured by a standard organism. When the streptomycin and streptomycin B used in the present studies were assayed against a streptomycin standard with *K. pneumoniae* as the test organism, the streptomycin employed had an activity of 650 u/mg and the various streptomycin B preparations had activities between 140 and 200 u/mg.† On this basis *E. typhosa* would require ca. 6 u of streptomycin to be inhibited in yeast beef broth, and ca. 1.5 u of streptomycin B. Hence, on a streptomycin unit basis, streptomycin B would appear to be more active than streptomycin against *E. typhosa*. This would also hold for the strain of *S. schottmülleri*,‡ as well as for a strain of *S. enteritidis*.§

It is of further interest to note that streptomycin B is not precipitated as a calcium chloride double salt[5] and, therefore, may well

[4] Hobby, G. L., and Lenert, T. B., The Action of Streptomycin *in vitro*. Conference on Antibiotic Research; Antibiotic Study Section of the National Institute of Health, Washington, D.C., Jan. 31-Feb. 1, 1947.

† With regard to the assignment of bioactivities, on the basis of streptomycin units, to antibiotics other than streptomycin itself, both the test organism and the test medium are of prime importance. Streptomycin bioassay procedures have been described in various laboratories using as the test organism any of a number of species of organisms and various test media. Even where a common streptomycin standard has been used, the units measured by the various procedures are equivalent only so long as streptomycin alone is present. If, on the other hand, an antibiotic other than streptomycin is to be bioassayed, the common streptomycin standard is of little use since the various test organisms may respond to the new antibiotic in entirely different fashions. This is well exemplified by comparing the data for *K. pneumoniae* and *Staph. aureus* (Osgood) in Table I. These two species are equally sensitive to streptomycin in yeast beef broth, but they differ significantly in their respective sensitivities to streptomycin B. Hence, the streptomycin unit loses its meaning when applied to antibiotics other than streptomycin.

‡ The authors wish to thank Mr. Otto Graessle of the Merck Institute for Therapeutic Research for the *S. schottmulleri* culture used here.

§ The strain of *S. enteritidis* referred to was tested in 0.75% tryptone broth, pH 8.5, but not in yeast beef broth, and therefore was not reported in Table I. In tryptone broth the M.I.C. of streptomycin for this organism was 0.079 γ/ml as compared to 0.187 γ/ml of streptomycin B, giving a streptomycin B/streptomycin ratio of 2.36.

[5] Fried, J., personal communication.

have been present in residues described by Hobby.[4] There is, therefore, reason to believe that the activity in residues referred to by Hobby may be due, at least in part, to the streptomycin B described by Fried and Titus.[1]

Effect of streptomycin B on an organism resistant to streptomycin. Unless special precautions are taken, even highly purified streptomycin preparations may contain some streptomycin B. Hence organisms made resistant to the usual streptomycin preparations may have been exposed also to streptomycin B and it would, therefore, not be surprising to find such organisms also resistant to the latter material. For the present work efforts were made to avoid such conditions in order to study whether development of resistance towards streptomycin was accompanied by resistance to streptomycin B. Hence, a freshly isolated strain of *E. coli,* highly sensitive to streptomycin, was made 25 times more resistant than originally by exposure to sublethal amounts of streptomycin free of streptomycin B. It was found that this organism had become similarly more resistant to streptomycin B. The lack of a preparation of streptomycin B entirely free of streptomycin, *per se,* prevented testing the reverse process, *i.e.* making an organism resistant to B and then testing its sensitivity to streptomycin free of streptomycin B.

II. *In vivo studies. A. Toxicity of streptomycin B in mice.* The intravenous toxicity of streptomycin B was compared with that of streptomycin in Swiss albino mice. Weight for weight the toxicities of the 2 materials were found to be very similar. Thus, a dose of ca. 5 mg/mouse (ca. 250 mg/kg) of either antibiotic caused severe shock and occasional immediate deaths. Higher doses (*e.g.* 7.0 mg/mouse) invariably caused immediate death while lower doses caused no deaths if the time taken for injection was at least 15 seconds. The appearance of mice in shock caused by either material was entirely similar and in neither case did any deaths or other signs or symptoms occur during a 21-day period following injection. Mouse weights and food intake data gathered during this period were normal and autopsies at the end of this interval showed no pathological changes.

B. Therapeutic action of streptomycin B. 1. In experimental tuberculosis in mice. Procedure. In experimental tuberculosis in mice the strain of mouse used is extremely important.[6] Studies (to be described elsewhere) as to the choice of mouse strain as well as the strain of *M. tuberculosis* to be used for such investigations as the present led to the use of a bovine strain of *M. tuberculosis* (Ravanel) and a strain of albino mouse known as the CF1 (Carworth Farms).

a. Inoculum. The Ravanel strain of *M. tuberculosis* was grown in a modified Kirchner's synthetic medium (described above). Inocula were usually prepared from a 5-day culture diluted to match the turbidity of a McFarland turbidity standard consisting of 0.0125 ml of 1% barium chloride solution plus 9.99 ml of 1% sulfuric acid. As a rule this meant diluting a 5-day culture 1/10 with physiological saline.

CF1 mice, weighing 16-18 g, were given 0.5 ml of the appropriately diluted culture intravenously. The dose per mouse was roughly equivalent to 0.6 mg of organisms (moist weight).

b. Treatment. Subcutaneous administration of streptomycin or streptomycin B was begun within an hour following the time of infection. A schedule of three 0.1 ml injections per day (morning, noon, and late afternoon) for 21 days was followed in the experiment reported here.

Results. In Table III is shown the comparative therapeutic action of streptomycin and streptomycin B. The antibiotic preparations used were similar to those described in the *in vitro* studies. Because of the small amounts of streptomycin B available, it was possible to have only 5 mice per dose in this group.

It will be recalled (Table I) that when tested *in vitro,* streptomycin was 3.7 times as active as streptomycin B (weight for weight) against *M. tuberculosis* H37Rv. It is evident from the data in Table III that a roughly similar relationship exists for the *in vivo*

[6] Pierce, C., Dubos, R. J., and Middlebrook, G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 173.

TABLE III.
Comparative Therapeutic Action of Streptomycin B and Streptomycin in Experimental Tuberculosis in Mice.

Antibiotic	Dose mg/kg/day	Dose u/kg/day*	No. of days treated	Deaths within 30 days	Avg survival time,† days
Streptomycin	49.45	32,100	21	0/10	
	12.36	8,025	21	9/10	25.0
	3.09	2,006	21	10/10	21.7
Streptomycin B	155.47	30,220	21	0/5	
	38.87	7,550	21	4/5	26.8
Untreated Controls				10/10	20.6

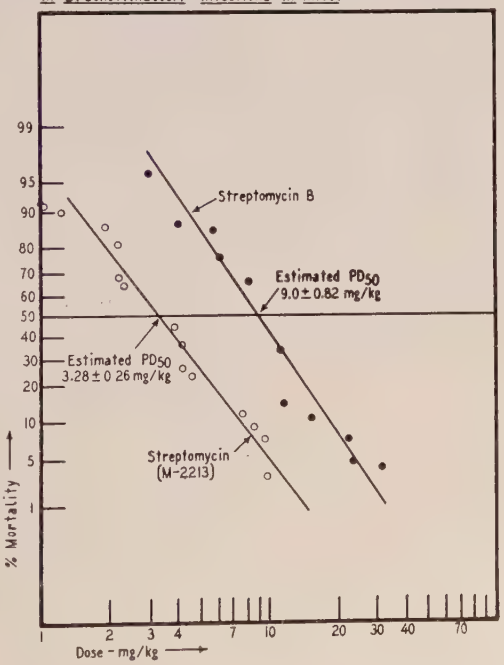
* The streptomycin used had an activity of 650 u/mg; that of the streptomycin B would be assigned on activity of 194 u/mg on the basis of assays with *K. pneumoniae* against a streptomycin standard.

† For purposes of calculation, mice surviving on the 30th day were assumed to have died on the 31st.

actions of these 2 antibiotics against the Ravanel strain in mice. Thus, using small numbers of mice complete protection for 30 days‖ was brought about by approximately ⅓ as much streptomycin as was required of streptomycin B. Since this is roughly the inverse ratio of the relative potencies which these 2 materials would have on the basis of units as measured by *K. pneumoniae* (650 u/mg as compared with 140-200 u/mg for streptomycin B) it can be seen that on a *unit basis* the 2 antibiotics would appear to be approximately equal in therapeutic action in experimental tuberculosis in mice. Final conclusions, of course, are not yet permissible since of necessity only small groups of mice were used in these studies and the steps in dosage range were 4-fold.

2. Comparative action of streptomycin and streptomycin B in experimental Salmonella schottmulleri infections in mice. Procedure. Sixteen-hour beef heart broth cultures diluted in 5% mucin were used for inoculating Swiss albino mice intraperitoneally. Mice to be treated received 1 ml, intraperitoneally, of a 1×10^{-7} dilution of a culture while control mice received 1 ml of dilutions ranging from 1×10^{-6}, through 10^{-9}. Plate counts of the cultures indicated that one organism per mouse proved fatal. Thus, in composite results of 5 tests the 10^{-7} dilution on the average contained 85 organisms per ml and killed 50/50 mice; 10^{-8} dilution (average 8.5 organisms per ml) killed 46/50 mice and 10^{-9} dilution (average 0.85 organisms per ml) killed 23/50 mice. Hence, the mice to be

— FIGURE I —
Comparative action of Streptomycin and Streptomycin-B in S. schottmulleri infections in mice.



‖ The dose levels used were adjusted to make comparisons possible at 30 days, but not high enough to prevent infection entirely. In other studies (to be described elsewhere) it was found that even doses of streptomycin 10 times as great as used here would not prevent eventual deaths of mice infected with tuberculosis.

treated received approximately 100 LD_{50}.

Treatment. Streptomycin and streptomycin B, in aqueous solutions, were administered in a single dose, 0.5 ml subcutaneously, within an hour after infection.

Results. Composite results of 3 tests with streptomycin and streptomycin B are shown in Fig. 1. In each test each therapeutic agent was tested at 4-dose levels, with 10 mice per level. Results, therefore, were calculated on the basis of response of 120 mice for each compound. For calculating the PD_{50} (dose protecting 50% of the mice), the composite data was first treated according to the method of Reed and Muench[7] and then plotted on probability log paper. Here the estimated PD_{50} was determined graphically and the standard error calculated by the method described by Miller and Tainter.[8]

The estimated PD_{50} for streptomycin was found to be 3.28 ± 0.26 mg/kg while that for streptomycin B was 9.0 ± 0.82 mg/kg. Hence, on a weight basis streptomycin was therapeutically approximately 3 times as active as streptomycin B. If one were to convert these weights to units as assayed with *K. pneumoniae* against a standard, the PD_{50} of streptomycin (at 650 u/mg) would be 2120 u/kg, and streptomycin B (at 170 u/mg) would be 1530 u/kg. Hence, on the unit basis now in use, streptomycin B would again appear to be more active than streptomycin.

It will be recalled, from the *in vitro* studies, (Table II) that in tryptone broth streptomycin was 1.23 times as active as streptomycin B, on a weight for weight basis, and 1.61 times as active as the latter in yeast beef broth against the same strain of *S. schottmulleri,* while it has now been shown that *in vivo,* streptomycin was ca. 3 times as active as streptomycin B. This may, of course, be due to differences in disposition of the 2 substances in the body, or perhaps, as suggested above, to differential effects of the body fluids on the activities of these 2 antibiotics. Final answer to this question must await, at least in part, on absorption-excretion studies with streptomycin B.

Summary. Streptomycin B, an antibiotic closely related to streptomycin, and separated from the latter by chromatography, has been studied *in vitro* against 8 strains or species of bacteria and in each case shown to be less active, on a weight basis, than streptomycin *per se.*

Toxicity studies in mice indicated that streptomycin B and streptomycin have approximately equal LD_{50} values on a weight basis.

In experimental tuberculosis infections in mice, streptomycin B was about ⅓ as active (again weight for weight) as was streptomycin, but was approximately equally active as the latter on the basis of presently accepted units.

In experimental *S. schottmülleri* infections in mice, streptomycin B again had ⅓ the activity of streptomycin on a weight basis, but would appear to be more active than the latter if comparison was made on a unit basis.

The authors wish to acknowledge the technical assistance of Mr. D. P. Canales in carrying out the experiments described in this paper.

[7] Reed, L. J., and Muench, H., *Am. J. Hygiene,* 1938, **27**, 493.

[8] Miller, L. C., and Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 261.

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Addiction Potentialities of 1, 1-Diphenyl-1-(β-Dimethylaminopropyl)-Butanone-2 Hydrochloride (Amidone) in the Monkey.*

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1,1-Diphenyl-1 - (β-dimethylaminopropyl)-butanone-2 hydrochloride (amidone, 10820, dolophine) has recently become the subject of intensive pharmacological studies as a result of its analgetic action. Information concerning this compound has been published by the U. S. Department of Commerce.[1] More recently reports have been published describing in greater detail the pharmacological actions of this derivative and its analgetic potency.[2,3] It is the purpose of this paper to present experimental data concerning the addiction potentialities of amidone in rhesus monkeys.[4] This animal is well suited for this type of study since the course of addiction and the signs of abstinence are similar to those observed in man.[5]

Chronically morphinized monkeys were used as a basis for comparison. Two rhesus monkeys were given morphine sulfate subcutaneously daily for 86 to 95 days, increased from 7.5 to 100 mg/kg in 50 days and maintained at this level. Four monkeys received amidone subcutaneously daily for 75 to 96 days, the initial dose of 5 mg/kg being increased to a maximum dose (13 mg/kg in the most resistant animals) in 24 to 26 days. The animals were maintained during the rest of the period on the maximum tolerated dose as determined by the severity of acute depression and weight loss. Pertinent data are summarized in Table I.

The chronically morphinized monkeys exhibited characteristic abstinence signs[5] after withdrawal of the drug. No characteristic signs of abstinence or evidence of increased irritability was observed in the monkeys receiving amidone. They returned to normal activity in a few days, appetite and weight loss were regained, and the response was entirely different from the morphinized animals.

Both morphine and amidone, when chronically administered, produce anorexia and weight loss (see Table I) to about the same extent. In addition, however, amidone causes considerable irritation and fibrosis at the site of injection resulting in marked aversion to daily administration.

Amidone, administered subcutaneously to the monkey, produces an acute depression which is maximum in 90 minutes and lasts 4 to 5 hours as an average response. Mydriasis, salivation, lacrimation, profound muscular weakness, and severe respiratory depression are characteristic signs, as with morphine. In the monkey the lethal dose of amidone lies between 10 and 20 mg/kg, death resulting from respiratory failure. Shortage of animals, which prevailed at the time of this study, prevented the establishment of a more accurate toxicity value.

With amidone, repeated administration does not confer tolerance to the lethal dose nor to other manifestations of depression. In fact, increased susceptibility is the rule as evidenced by the fact that profound depression required a reduction in dosage below that previously tolerated.

In order to determine if any crossed tolerance from amidone to morphine had devel-

* Supported by a grant from Parke, Davis and Company.

1 Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report No. 981, Office of the Publication Board, Department of Commerce, Washington, D.C.

2 Scott, C. C., and Chen, K. K., *Fed. Proc.*, 1946, **5**, 201; *J. Pharm. Exp. Therap.*, 1946, **87**, 63.

3 Scott, C. C., Robbins, E. B., and Chen, K. K., *Science*, 1946, **104**, 587.

4 Woods, L. A., Wyngaarden, J. B., and Seevers, M. H., *Fed. Proc.*, 1947, **6**, in press.

5 Seevers, M. H., *J. Pharm. Exp. Therap.*, 1936, **56**, 147.

TABLE I.
Dosage and Weight Data of Chronically Poisoned Monkeys.

Monkey No.	Sex	Initial wt (kg)	Loss in wt (%)	Drug	Days of injection	Max. dose tolerated mg/kg
40	M	4.8	10.4	M.S.	95	—
45	F	5.5	19.1	"	86	—
27	F	4.3	30.2	Amid.	96	13
28	M	5.0	18.0	"	96	13
42	M	5.0	10.2	"	75	11
46	M	5.6	7.0	"	75	12

oped, the 4 amidone monkeys (on the 73rd day for monkeys No. 27 and No. 28 and 53rd day for monkeys No. 42 and No. 46) and 4 normal monkeys were injected with morphine sulfate 20 mg/kg at approximately the same time and careful observations and comparisons noted. The depth and duration of depression, and the degree of lacrimation, mydriasis, and salivation and other signs were not significantly different for the 2 series of animals, indicating that chronic poisoning with amidone does not confer tolerance to morphine.

Observations on the chronically morphinized monkeys, however, did indicate some, but not marked, crossed tolerance to amidone. Monkeys No. 40 and No. 45 were each given 15 mg/kg of amidone at 2 different times separated by a week's interval. Each of these 4 injections produced only a mild to moderate depression. Injection of monkey No. 40 with 18 mg/kg of amidone produced a moderately severe depression. During these injections of amidone, although 48 hours elapsed between morphine sulfate administration, no withdrawal signs appeared. Five normal control monkeys were injected with 15 mg/kg of amidone and the effects were more pronounced than those seen in the morphinized animals since: one monkey died in spite of artificial respiration; one monkey was very severely depressed but the animal survived after artificial respiration and administration of metrazol; 2 monkeys were very depressed with very slow respiratory rate and Cheyne-Stokes breathing. Only one of this group of 5 exhibited mild to moderate depression, comparable to that produced by a similar quantity of amidone in the chronically morphinized animal.

Summary. It may be stated that chronic administration of amidone in the monkey does not lead to a condition of physical dependence on this drug such as is noted with morphine. Tolerance to this drug does not develop, but on the contrary an increased susceptibility to its depressant effect occurs. Crossed tolerance from amidone to morphine does not occur but chronically morphinized monkeys do exhibit some crossed tolerance to amidone. The later is only minimal and not comparable in degree to that which occurs from one morphine derivative to another. Amidone produces an acute depression which is maximum in 90 minutes and is of 4 to 5 hours' duration in this animal. The lethal dose in the monkey lies between 10 and 20 mg/kg and death occurs from respiratory failure.

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Effect of Wheat Gluten Diet on the Electroencephalograms of Dogs.*

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Introduction. Wagner and Elvehjem[1] have shown that "running fits" or so-called "canine hysteria" can be induced in dogs by feeding a ration containing wheat gluten. Convulsive seizures, typical of running fits, suggested the possibility that this condition might be related to epilepsy.[2] It seemed of interest, therefore, to determine the electroencephalogram changes, if any, which might be induced by wheat gluten feeding.

Method. Mongrel puppies ranging in age from 6 to 8 weeks were used in this work. Each animal was dewormed and dusted with D.D.T. before the beginning of the experiment. For a period of a week or more before the operation and during the period when normal electroencephalograms were taken the animals were maintained on a purified basal control ration.†

Under general anesthesia with phenylethyl-barbarturic acid (Nembutal), 4 tantalum electrodes were placed extradurally about 10 mm posterior to the cruciate sulcus and 5 to 10 mm from the midline or just medial to the upper margin of the temporal muscles. The technic used here was the same as is used in clinical electroencephalography except that the electrodes were anchored firmly to the bone in order to minimize movement.[3] This was accomplished by twisting the wires over the bridge of bone between 2 adjacent holes in the skull. All records were taken without the use of any type of anesthesia or drug. Electroencephalograms were made on the dogs with a 6-channel recorder. However, records from only one channel are presented here as they are representative of the results. Wheat gluten was substituted for casein in the ration after several satisfactory normal records were obtained. Generalized convulsions were frequently observed after 3 to 10 days on the gluten ration; and often there was evidence of their occurrence, such as upset food containers and disturbance of the shavings in the cage.

Results. Eighteen dogs have been tested in this series of experiments. However, the data on only one animal are included here as they are representative of the results obtained. Electroencephalogram (E.E.G.) abnormalities were noted as early as 3 days after a dog was placed on a wheat gluten ration, while the maximum time for E.E.G. changes to occur in the animals studied was 24 days. Running fits have been observed in as short a time as 3 days while gluten has been fed for as long as 5 weeks during which period no epileptic seizures were observed. Variation in response seemed to depend somewhat upon the age and breed of the dog used. Data indicate that the E.E.G. changes appeared before any clinical abnormality was noted.

The control E.E.G. record (Fig. 1, July 28), made while the 7-week-old male puppy

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1 Wagner, J. R., and Elvehjem, C. A., *J. Nutrition*, 1944, **28**, 431.

2 Erickson, T. C., Gilson, W. E., Elvehjem, C. A., and Newell, G. W., *Annual Volume on Epilepsy*, 1946, presented December 14, 1946, to a meeting of the Association for Research in Nervous and Mental Disease, New York City.

† Casein 20, sucrose 69, salts IV 4, corn oil 5, whole liver powder 2. Oral supplements of 0.75 mg thiamine, 0.75 mg riboflavin, 0.5 mg pyridoxine, 0.25 mg pantothenic acid, 1 mg nicotinic acid, and 70 mg choline per kilo of body weight, and 4 drops of haliver oil were administered twice weekly.

3 Gibbs, F. A., *Medical Physics*, p. 361, Year Book Publishers, Inc., Chicago, Ill.

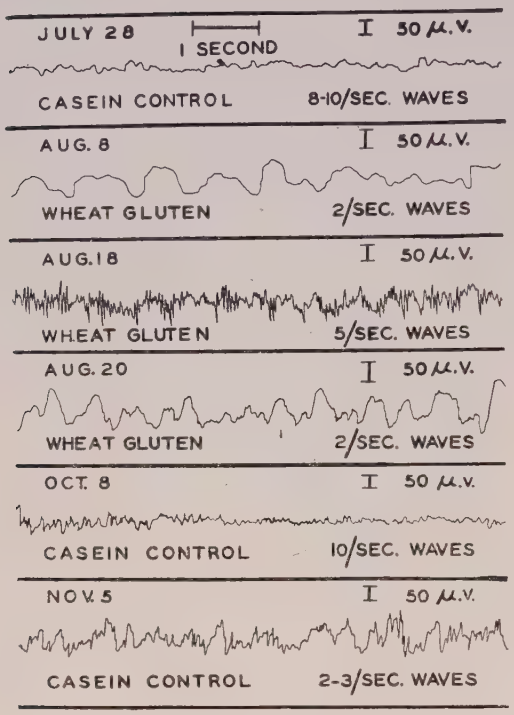

FIG. 1.

used in this experiment was on the basal ration, showed a moderate voltage activity with a predominant frequency of 8-10 per second. This same day the ration was changed to include wheat gluten in place of the casein. Eight days after this new ration had been fed the dog exhibited no change in behavior and a similar voltage activity and frequency resulted as was obtained with the control ration.

On August 8, 11 days after beginning gluten feeding there occurred a definite change in the electroencephalogram with the appearance of high voltage slow waves at a frequency of 2 per second. Three days later the same type of record was obtained although no outward change in the condition of the dog could be observed. On August 18, a series of convulsions occurred and the record for this date was taken about 2 minutes after one of the convulsions had terminated. Here many spikes can be seen with some low voltage waves at a frequency of 5 per second. This record, taken immediately after a generalized tonic clonic convulsion, shows a typical seizure wave pattern. A record taken 2 days later, on August 20, shows occasional 1.5 to 2 per second high voltage waves. Both the seizure and interval E.E.G. were comparable to those seen in human epilepsy.

The following day, August 21, the casein control ration replaced the one containing gluten. On October 8, 7 weeks after the dog had been fed the casein control ration the E.E.G. showed a normal pattern with moderate voltage activity and 8-10 per second waves. After casein replaced gluten in the ration no further seizures were observed. Another record made 3 days later, not shown here, again showed a normal type of wave pattern. A check E.E.G. made one month later, November 5, showed a reversion to the type of wave pattern seen when the animal was fed the gluten. Occasional spikes are evident here with 2-3 per second wave activity. This fluctuating abnormality in the E.E.G. after gluten feeding was terminated has been observed in several dogs. In some animals seizures have been noted as long as 5 days after gluten was discontinued.

Summary. A method of obtaining electroencephalograms in dogs has been described. When rations which contained casein were fed no abnormalities were observed. After wheat gluten was substituted for casein epileptiform seizures and concomitant abnormalities in the E.E.G. developed which were comparable to those seen in cases of human epilepsy.

15884

Improvement of Vitamin A Deficient Diets for Use in Bioassays.

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The use of vitamin A-free diets of the U.S.P. (United States Pharmacopoeia) and B.P. (British Pharmacopoeia) types for biological assay of foods and feeds possessing vitamin A activity has been questioned recently by various investigators. They have pointed out that these diets do not permit normal growth in rats even when a generous amount of vitamin A is supplied as a supplement. Several workers have, in turn, proposed modifications in, or entirely new formulas for, such vitamin A-free diets. Gridgeman[1] has cited some of this work.

From the standpoint of the improvement of purified diets fortified with all of the known vitamin B factors, enhanced growth in rats has been obtained both by the use of liver extract and by increased level of casein in the diet. In the first instance, Hartman and co-workers[2,3] have conducted numerous experiments dealing with a growth factor lacking in diets such as the U.S.P. vitamin A assay diet but present in a wide variety of natural products. They have prepared highly active concentrates of the factor from liver extract which promote enhanced growth in rats maintained on the usual synthetic diet used in vitamin A assay. That casein may carry varying amounts of the factor is shown by differences in growth obtained with different casein preparations as purified and used in various laboratories, commercial and otherwise. McIntire *et al.*[4] also suggested that liver extract supplied a missing factor in, or otherwise corrected, a deficiency condition. Bosshardt *et al.*[5] have concluded that extracts of liver and pancreas contain a growth-promoting substance for mice which is distinct from the recognized B factors and not present in yeast in appreciable amounts. Zucker and Zucker,[6] on the other hand, obtained improved growth by increasing the casein content from 18 to 28% or thereabouts and suggest that the missing factor "is associated whether as an impurity or an essential amino acid, with protein of good quality." Deuel *et al.*[7] have obtained better growth by substitution of mackerel protein for casein in the U.S.P. vitamin A assay diet.

The foregoing evidence plus other accessory facts seems to show rather definitely that the U.S.P. XII diet lacks some growth factor or factors other than vitamin A. The work reported here is the result of experiments designed to include this new factor in the vitamin A-free diet. Although the substance has not been isolated as a pure compound, it is possible to include it in the form of natural products, while still maintaining a vitamin A-free diet. This may not be fully satisfactory, but should contribute to accuracy in the assay of many materials.

Experimental. The feeding tests were conducted with male and female rats, 21 to 26 days of age. Equal numbers of the 2 sexes were used in each group and the rats were further distributed in the groups as evenly as possible with regard to age, weight, and genetic history.

Modifications were made in the basal vitamin A-deficient diet, regularly used in the

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6 Zucker, T. F., and Zucker, L., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 136.

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TABLE I.
Composition of Rat Diets.

Ingredients*	% of ingredient in diet No.			
	568	687	747	748
Casein	18	9	9	9
Pork muscle		9		
Sardine meal			12	
Blood fibrin				9
Dextrin	66	66	63	66
Yeast, non-irr.	7	7	7	7
" irr.	1	1	1	1
Salt mixture	3	3	3	3
Peanut oil	5	5	5	5

* Alpha tocopherol was added to all diets at the rate of 3 mg per 100 g.

laboratory and essentially the U.S.P. XII diet, to determine the effects produced by the use of various materials known to contain the growth factor already discussed. One of these materials was lean pork obtained from fresh hams. The pork was first cooked, then dehydrated, extracted with ethyl ether in a percolator, and finally ground. Blood fibrin was also prepared for use by grinding and extracting with ethyl ether. The third material used as a substitute for casein was ether-extracted fish (sardine) meal. The casein as used in vitamin A feeding tests in this laboratory is first extracted 3 times with acidulated water and then with 95% alcohol (hot) for 24 hours in a large metal Soxhlet extractor.

The composition of the basic experimental diets is given in Table I. In the feeding tests, groups of rats were fed the diets as given in Table I. Rats on diets 747 and 748 also received orally 0.05 ml of liver extract 3 times per week. Four parallel groups received the same 4 diets plus a supplement of cod liver oil (series A) to supply vitamin A at a level of 50 I.U. per rat per day. Another (series B) received the cod liver oil as in (series A) plus liver extract fed by dropper to each rat at the rate of 0.05 ml 3 times per week. As a control, one group of rats received the complex stock colony diet made up mainly of natural and other concentrate feeds.

Results. Preliminary experiments with a diet of the U.S.P. type such as diet 568 in Table I supplemented with vitamin A, showed that increasing the extracted casein from 18% to 23% and adding 0.2% of *l*-cystine failed to produce increased growth. The animals averaged little more than 15 g per week over an 8-week growth period, while animals fed a stock colony diet averaged nearly 25 g per week. This seemingly indicated that the casein and yeast furnished adequate protein, but failed to provide some other necessary factor or factors.

Feeding results of the comparisons of the diets given in Table I are presented in Table II. That the basal casein diet, plus vitamin A (diet 568A), is deficient for optimum growth is indicated plainly by the relatively poor mean gains over a period of 8 weeks. This deficiency in growth appears to have been corrected by addition of 0.05 ml of the liver extract, since the difference in growth on diet 568B and on the stock colony diet is probably not significant. Increased growth was also obtained in diet Series A and B with extracted pork muscle, sardine meal, and blood fibrin. The best gains were made on the supplemented sardine meal diets. While a complete statistical analysis of the data has not been made, it appears evident that the sardine meal was superior to the pork. Both supplied the growth factor contained in the liver extract since the addition of the latter material in diet treatments 747B and 748B but omission in 747A and 748A had little if any effect on growth. That sardine meal, pork muscle, and liver extract were free of vitamin A activity was shown by the animals fed diets 687 and 747. All of the animals on these diets showed severe symptoms of vitamin A deficiency, being just as

TABLE II.
Results of Rat Feeding Tests Lasting 8 Weeks.

Diet treatment	Diet No.	No. animals		Avg wt gain (g)
		Start	Close	
Basal casein diets				
No supplement	568	8	5	46.2
+ 50 I.U. of A*	568A	8	8	135.3
+ .05 ml L.E.† and 50 I.U. of A.	568B	14	14	172.4
Pork diets				
No supplement	687	8	3	60.0
+ 50 I.U. of A.	687A	8	8	162.0
+ .05 ml L.E. and 50 I.U. of A.	687B	8	8	163.4
Sardine meal diets				
+ .05 ml L.E.	747	5	1	28.0
+ 50 I.U. of A.	747A	6	6	191.8
+ .05 ml L.E. and 50 I.U. of A.	747B	6	6	193.1
Blood fibrin diets				
+ .05 ml L.E.	748	6	6	181.0
+ 50 I.U. of A.	748A	6	6	192.8
+ .05 ml L.E. and 50 I.U. of A.	748B	6	6	190.2
Stock colony diet				
No supplement	—	14	14	183.7

* A—vitamin A.
† L.E.—liver extract.

pronounced as on diet 568. This was not true of the animals fed diet 748, indicating that extraction failed to remove A-activity from the blood fibrin.

It should be noted that the known B complex factors are furnished in the diets given in Table I by the 8% of high potency yeast. The work reported by Hartman and Cary[3] along with unpublished data obtained in this laboratory show that the addition of all of the known B complex vitamins, including pteroyl glutamic acid, to this type of diet will not produce normal growth. Accordingly it seems evident that the results reported here show that the U.S.P. type of diet is deficient in at least one unknown factor. The data further indicate means by which the diet may be made complete so that it can be used in obtaining more satisfactory vitamin A assays.

Summary. Results of experiments are given which show that for optimum growth in rats, it is necessary to add at least one factor besides vitamin A to the U.S.P. vitamin A-free diet. This factor, which has been described by other investigators, is present in liver extract. Replacement of half of the casein in the basal diet either with pork or sardine meal was likewise effective and the addition of liver extract to these ingredients did not give any added benefit. Unfortunately, extraction with ether failed to remove the vitamin A from blood fibrin to make possible its use. Excepting the groups fed blood fibrin the sardine meal diet with supplements promoted the best gains of all the remaining groups.

15885

Calcium Enriched Meat Compared with Milk as Source of Calcium Phosphorus and Protein.

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Not infrequently need arises for an efficient substitute for milk as a source of calcium in the diet of infants and growing children. Complete substitution is often necessary for those who are allergic to milk proteins and for those who are maintained on a ketogenic diet extremely low in carbohydrate as a means of therapy for either epilepsy or a urinary tract infection. Many children tire of milk early in life and so fail to consume enough of this food item to satisfy their daily requirements for calcium. In some parts of the world milk is entirely unavailable to large sections of the population, making a suitable substitute desirable.

Although many green vegetables, such as spinach, chard and beet greens, are as rich in calcium as milk on a dry-weight basis, they do not constitute a satisfactory food source of this element because much of the calcium occurs in already unavailable form or combines in the gastrointestinal tract with oxalic acid contained in the plants to form insoluble calcium oxalate.[1] The calcium of fresh carrots, lettuce and string beans[2] and whole soy beans[3] is far less available than that of milk, no matter how thoroughly the vegetables are cooked. It has been demonstrated that the percentage utilization of the calcium in di-calcium phosphate[4] and in calcium gluconate[5] is approximately the same as that of milk calcium, but attempts to induce young children to satisfy their calcium needs over long periods of time by consuming such salts directly rarely proves satisfactory. It has been reported that lactose, in contrast to other carbohydrate foods, increases calcium utilization significantly in growing children and lower animals.[6]

The present study was undertaken to compare the nutritional value of a calcium-enriched ground beef diet with that of milk from the viewpoint of calcium, phosphorus and protein utilization. By various additions the 2 diets were adjusted to contain the same quantities of protein, fat, carbohydrate, water, calcium, phosphorus and vitamins so far as this could be done. Two boys, 12 and 14 years of age respectively, who had been maintained on adequate hospital diets furnishing more than 1 g of Ca daily for several weeks were placed on the milk-containing diet for 15 days and then on the Ca-enriched meat diet for 12 days while Ca, P, and N_2 balance were being determined. The subjects were comparatively inactive, neither being able to walk. The 12-year-old boy was partially incapacitated by progressive muscular dystrophy. The 14-year-old boy had suffered from extensive poliomyelitis of both lower extremities 2 years previously. During these 2 periods the daily diet contained 2.35 g of protein, 45 mg of Ca and 41 mg of P per kg of body weight. Following the second period, the protein was reduced to 0.5 g per kg of body weight daily without change in the Ca and P intake for a period of 16 days to determine the effect of different levels of dietary protein on Ca and P absorption and excretion.

1 Wohl, M. G., *Dietotherapy*, Saunders, Philadelphia, 1945, Chapter 8 by Holmes, J. O.

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3 Shroeder, L. J., Cahill, W. M., and Smith, A. H., *J. Nutrition*, 1946, **32**, 413.

4 Kempster, E., Breiter, H., Mills, R., McKey, B., Brends, M., and Outhouse, J., *J. Nutrition*, 1940, **20**, 279.

5 Steggerda, F. R., and Mitchell, H. E., *J. Nutrition*, 1939, **17**, 253.

6 Mills, R., Breiter, H., Kempster, E., McKey, B., Pickens, M., and Outhouse, J., *J. Nutrition*, 1940, **20**, 467.

Young litter-mate rats about 35 days of age were divided into 2 dietary groups, one receiving milk, the other cooked lean meat with added Ca gluconate. Data on the males and females were kept separately. Three groups of animals were maintained on the meat diet and 3 on the milk diet for 6 weeks. Another group in each category was kept on the diet for 8 weeks.

At the end of the period the animals were killed by decapitation 24 hours after all food had been removed from the cages. Blood samples from individual rats in each group were pooled for serum protein, Ca and P determinations. The femurs alone were weighed and analyzed in approximately half of the rats on each diet. The entire carcass of each of the remaining rats was analyzed for total fat-free dry weight and for Ca and P.

Results. The serum protein, Ca and P levels at the end of the various experimental periods showed no significant differences attributable to differences in diet in either the rats or the human subjects. All values were well within normal range.

Despite the comparatively high Ca intake, each of the 2 boys showed a slightly negative Ca balance with both diets, when 2.35 g protein per kg was given daily. At the same time, the P and protein balances were positive. The average per kg daily balances for the 12-year-old boy expressed in mg were as follows: Milk diet: N_2 +51; P +2.3; Ca —5.1; meat diet: N_2 +73; P +5.5; Ca —3.6. Corresponding figures for the 14-year-old boy were as follows: Milk diet: N_2 +63; F +2.8; Ca —4.2; meat diet: N_2 +76; P +6.9; Ca —1.7. Corresponding figures for each subject on the meat diet with 0.5 g protein per kg instead of 2.35 g were as follows: 12-year-old boy: N_2 —13.8; P +5.6; Ca —7.1; 14-year-old boy: N_2 —1.8; P +13.8; Ca +3.6.

The retentions of N_2, Ca and P by the rats as judged by analyses of the femurs alone or by the whole carcass were found to be equally as good for the groups given the pureed cooked beef muscle mixed with Ca gluconate and phosphates as for those given milk solids as the source of these constituents. During the experimental period the meat-fed rats (33 animals) showed an average increase of 206% over the initial weights, whereas the average increase for those receiving the milk diet (31 animals) was 172%. The total caloric intake per day averaged 46 for the meat-fed and 41 for the milk-fed rats.

The average wet weight of the cleaned femurs of the meat-fed rats was 615 mg and that of the milk-fed animals was 583 mg. Averages for the a. total dry-weight, b. fat-free dry-weight, c. organic matter and d. total ash of the femur of the meat-fed animals were as follows: a. 357; b. 348; c. 129; d. 228 mg. Corresponding average figures for the femur of the milk-fed rats were as follows: a. 306; b. 302; c. 115; d. 202 mg.

Whole carcass ash analyses showed the following values per 100 g wet body weight: meat-fed (22 rats): average per rat 3.67 g total ash containing 29.6% Ca and 16.9% P; milk-fed (23 rats): average per rat 3.82 g total ash containing 27.8% Ca and 16.7% P. In one group of 6 meat-fed rats whose diets were made to contain the same amount of lactose as that in the milk diet the total ash per rat averaged 3.82 g as against 3.58 g ash in the nonlactose-containing meat diet. Throughout the study, the average values for male animals were consistently larger than those for females, excepting in percentage composition.

Summary. The nutritional value of a Ca-enriched meat diet has been investigated from the viewpoint of protein, Ca and P utilization. This has been compared experimentally with a milk diet containing the same quantities of essential food constituents. Balance studies were carried out in 2 young hospital patients. Total carcass and separate femur analyses were also made in rats on the 2 types of diet. The results of these studies indicated clearly that the Ca-supplemented meat diet was equally good as the milk diet as a source of Ca, P, and protein.

15886

Effect of Electrically Induced Convulsions on Rate of Alcohol Metabolism in Man.

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It is well established that the rate of metabolism of alcohol, as assessed from its rate of disappearance from the blood stream, is peculiarly resistant to change in a given individual. However, certain substances, notably insulin,[1] some amino acids,[2] and pyruvic acid[3] have been shown to exert an appreciable acceleration of alcohol metabolism, the increase in rate ranging upwards to 50%. Because of the considerable metabolic changes incidental to electrically-induced convulsions,[4] it was felt that the effect of this procedure on the rate of alcohol metabolism warranted investigation.

To this end, the rate of alcohol metabolism in 6 patients suffering from functional mental disease was studied prior to and immediately after institution of treatment with electric shock. The dose of alcohol was 1.5 cc per kg of body weight, administered intravenously as a 20% solution in normal saline, a period of one hour being required for the injection into a cubital vein. Two hours were then allowed to elapse to insure equilibration of the alcohol between the blood and tissues. Samples of blood were then taken at hourly intervals and their alcohol content determined by the method of Newman and Abramson.[5]

The above procedure was followed in all cases before shock treatment was instituted, and constituted the control observation. From the blood alcohol values so determined the rate of disappearance of alcohol from the blood over any hourly period could be computed, and these values for the first hour and the average for the first 2 hours after the period allowed for equilibration are set forth in Table I.

On a subsequent occasion the identical procedure was repeated, except that at the end of the period of equilibration, and immediately after securing the first blood specimen, a generalized convulsion was induced in the customary manner by passage of 60-cycle alternating current between 2 electrodes placed one on each side of the head in the temporal region. The values determined for rate of disappearance of alcohol from the blood were computed as for the controls, and

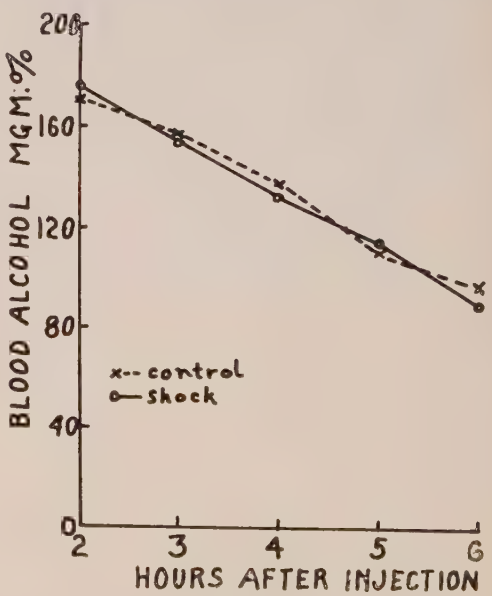

FIG. 1.

Rate of decline of blood alcohol concentration in control and after electrically induced convulsion. The shock was administered 2 hours after the completion of the injection.

[1] Clark, B. B., Morrissey, R. W., Fazekas, J. F., and Welch, C. S., *Quart. J. Studies on Alcohol*, 1941, **1**, 663.

[2] Eggleton, M. G., *J. Physiol.*, 1940, **98**, 239.

[3] Westerfeld, W., Stotz, E., and Berg, R. L., *J. Biol. Chem.*, 1942, **144**, 657.

[4] Lowenbach, H., and Greenhill, M. H., *J. Nerv. and Ment. Dis.*, 1947, **105**, 343.

[5] Newman, H. W., and Abramson, M., *J. Pharm. and Exp. Therap.*, 1942, **74**, 369.

TABLE I.
Rate of Decline of Blood Alcohol Concentration in mg per 100 cc per Hour, With and Without Convulsion.

Subj.	First hr			First 2 hr		
	Control	Shock	Change	Control	Shock	Change
Sch.	27	12	—15	23	18	—5
Rag.	12	22	+10	16	22	+6
Doa.	20	27	+ 7	21	23	+2
Mc.	15	19	+ 4	13	16	+3
Cro.	18	23	+ 5	23	24	+1
Mos.	28	22	— 6	20	18	—2
Mean change			+ 0.9			+0.9
Stand. dev.			8.8			3.6

appear in Table I. Fig. 1 is a graphic representation of the rate of fall of blood alcohol concentration in one patient with and without convulsion.

Examination of Table I shows that the average rate of decline of blood alcohol concentration was slightly greater after electric shock than when no convulsion was induced. However, it also shows that these differences are very small in comparison with the standard deviation, and thus are certainly not of statistical significance. Ziskind,[6] working with rabbits, has found a similar lack of effect of shock on rate of fall of blood alcohol concentration.

For fear that we might be missing some precipitate drop of short duration occurring immediately after the convulsion, in several of the cases a blood sample was secured 15 minutes and 30 minutes after shock. These revealed no more evidence of acceleration than did the samples taken at longer intervals.

We conclude that electrically-induced convulsions are not effective in significantly influencing the rate of alcohol metabolism in man.

6 Ziskind, E., personal communication, 1947.

15887

Antibody Response of Human Beings to Centrifuged, Lyophilized Japanese B Encephalitis Vaccine.*

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Since 1945, more than 350,000 people[1] have been inoculated with Japanese B encephalitis vaccine prepared in the U.S.A.[2] Since 1942, a number of studies have been carried out on the antibody response of human beings to various dosages and preparations, in an attempt to determine the proper dose in human beings particularly in relation to the potency of the vaccine as determined by assay in mice. Studies carried out without such reference to the quantitative determination in mice of the antigenic potency of the vaccine used for the human beings provide no useful information for comparative purposes, since vaccines prepared and stored in

* Aided by the Commission on Neurotropic Virus Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

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1 Sabin, A. B., *J. A. M. A.*, 1947, **133**, 281.

2 Sabin, A. B., Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruehman, I., *J. A. M. A.*, 1943, **122**, 477.

TABLE I.

Data on Japanese B Encephalitis Vaccine Used in Tests Recorded in Table II.

Preparation: 7/24/42– 10% mouse brain suspension in physiological salt solution, centrifuged on Swedish angle centrifuge at about 3,000 r.p.m. for 30 minutes. Titer of virus in centrifuged material before addition of formalin to a concentration of 0.2% was $10^{-8.4}$ (per 0.03 cc).
7/26/42—Vaccine lyophilized; tests for active virus negative; preliminary assay for immunogenic potency.
7/27/42—Lyophilized vaccine stored in refrigerator at 2°-3°C.
8/25/42—Vaccine assayed in mice and human volunteers inoculated.

"Long" Assay in 3- to 4-week-old Mice—Single Dose on 8/25/42—Challenge Test, 9/1/42.

Amount of vaccine cc	0.3 cc of indicated dilution of virus injected intra-abdominally									LD_{50} titer reciprocal log of dilution	Immunity index
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
None	9/10*	7/10	6/10	5/10	7/10	3/10	3/10	0/10	0/10	5.6	—
0.3	4/8	0/6	0/5	0/5	1/6	0/6	0/6			2.1—?	3,200+?
0.03	2/8	0/3	2/4	1/5	1/5	0/6	0/6			2.0—?	4,000+?
0.003	5/9	2/6	2/4	1/5	2/5	2/5	1/6			3.5	125

* Numerator = No. died of encephalitis; denominator = No. inoculated.

"Short" Assay in 10-week-old Mice.

10/16/42—first dose of vaccine; 10/19/42—second dose. 10/23/42—challenge with 0.3 cc of 10^{1} virus suspension intraäbdominally. Intracerebral titer of challenge virus = $10^{-8.6}$ per 0.03 cc.

Amt of vaccine	Result of challenge inoculation
None	8/12
(0.15 × 2) — 0.3 cc	0/12
(0.015 × 2) — 0.03 cc	3/10
(0.005 × 2) — 0.01 cc	2/10

50% immunogenic dose = <0.01 cc

different ways can vary markedly in their immunogenic properties. In mice the development of significant, although occasionally small, amounts of neutralizing antibodies, which generally follows vaccination, has been found by us invariably to be associated with demonstrable resistance to infection by the intra-abdominal route. Although vaccination may give rise to a certain amount of resistance to infection without demonstrable antibodies, it appeared desirable that the dosage of vaccine selected for human beings should be capable of producing neutralizing antibodies at least in an appreciable proportion, even if not in all, of the inoculated people.

The first series of tests carried out in June, 1942 on a group of 6 people associated with the laboratory gave confusing results, first, because each one of the sera taken before vaccination had varying, but definite, amounts of neutralizing antibody, and secondly, because the vaccine used was prepared by a method which was subsequently found to be unsatisfactory. The formalin in the centrifuged vaccine was neutralized with NH_4OH one week after preparation, and it was stored in the frozen state in an insulated box with solid CO_2 for 24 days prior to its use in human beings and rhesus monkeys. An assay carried out simultaneously in mice showed a distinct loss in the antigenic potency of the stored vaccine. The second series of tests was carried out in August, 1942, with a lyophilized vaccine[2] which was stored in an ordinary refrigerator at 2 to 3°C. The data on this vaccine are given in Table I. Although the method of assay subsequently developed by one of us (A.B.S.) as the standard for the National Institute of Health was not available in 1942, it may be seen from the data shown in Table I that the vaccine inoculated into the second series of human volunteers had a 50% immunogenic dose (ID_{50}) within the range of the 0.01 cc which was subsequently adopted as the minimal requirement for an acceptable vaccine. The 12 human volunteers for this test were selected from among a group of 29 medical students because their sera were found to contain no antibodies for the Japanese B virus. These students were bled a second time just before injection of the vaccine as an additional check on the absence of antiviral substance in their blood prior to vaccination. The schedule of injections and the dosage are indicated in Table II. Further blood specimens were obtained 1 week, 2 weeks, and 4 weeks after injection of the first dose of vaccine. The sera were tested either in the fresh state, or when that was not practical, they were stored in the frozen state in an insulated box containing solid CO_2.

The data presented in Table II indicate that the angle-centrifuged, lyophilized vaccine, in single or multiple doses of 4 to 6 cc, was capable of giving rise to antibodies for the Japanese B encephalitis virus, but not in all inoculated individuals. The small numbers of volunteers in each group do not permit any conclusions regarding the relative merits of the same amount of vaccine administered in single or multiple doses. There was considerable question as to how to interpret the neutralization indexes in the range of 16 to 40 which appeared in individuals whose sera obtained prior to vaccination had indexes not greater than 3, and the tendency was to regard them as indicative of definite, though small, amounts of antibody. Two sera, obtained 4 weeks after vaccination, from students "Prit." and "Ulev." in the 3-dose group, which on repeated tests gave these equivocal intracerebral neutralization indexes, were tested in an intra-abdominal neutralization test in 2-week-old mice with completely negative results. In work done by one of us (A.B.S.) 3 years later it was found that in sera of patients with Japanese B encephalitis, an intracerebral neutralization index of 20 to 30 may be equivalent to an intra-abdominal neutralization index of 10,000 to 500,000.[1] It is apparent, therefore, that it may be best to continue to classify intracerebral neutralization indexes over 10 and under 50 as equivocal, and to disregard them in the final evaluation of the serological response to vaccination. On the basis of this criterion, only one of the 12 volunteers developed antibodies within one week, and 4 within 2 weeks; the

TABLE II.

Neutralizing Antibodies for Japanese B Encephalitis Virus in Medical Students Inoculated with Angle Centrifuged, Lyophilized Vaccine.

Dosage and dates	Name	Intracerebral neutralization index				
		Before vaccine		After vaccine		
		Bleeding 1 8/13/42	Bleeding 2 8/25/42	1 week 9/1/42	2 weeks 9/8/42	4 weeks 9/22/42
One dose	Bail.	3	3	10	**400**	**80**
4 cc	Cowg.	—2	3	*32*	*25*	5
8/25/1942	Bast.	1	1	4	1	3
	Earl.	—2	4	5	8	4
Two doses	Scha.	—1	3	**100**	**50**	**80**
2 cc each	Gill.	—2	1	5	8	3
8/25/1942	Schi.	1	3	1	2	2
28	Frie.	—2	1	1	—2	4
Three doses	Down	2	3	8	**2,500**	**800**
2 cc each	Jaco.	4	3	3	**2,500**	**3,200**
8/25/1942	Prit.	—1	3	*16*	*25*	10; *25*
28	Ulev.	1	—2	*25*	*40*	*40*; *25*
31						

Aliquot portions of the same preparation of virus frozen in separate ampoules were used in all the tests. The neutralization indexes were calculated from the combined LD_{50} titer of this virus preparation in control rabbit serum mixtures of many different tests, which was $10^{-8.2}$. All the sera shown in this table were included in 5 tests in which the LD_{50} titers of the controls were $10^{-8.3}$, $10^{-7.8}$, $10^{-8.5}$, $10^{-8.0}$ and $10^{-8.0}$. The prevaccination sera of 8/25/42, and the 1-week and 2-week postvaccination sera were tested simultaneously. The neutralization indexes not preceded by a minus sign represent the ratio of the control LD_{50} titer to that of the serum; when the titer of the serum was greater than that of the control, the ratio was reversed and the result recorded as a negative quantity preceded by a minus sign. The values which are not in boldface or italics are regarded as negative because similar variations which are inherent in the test can be obtained with the same serum and virus preparation; the values in italics are regarded as equivocal, and those in boldface as positive.

4 individuals with antibodies at 2 weeks, were still positive 4 weeks after vaccination.

Summary. Neutralizing antibodies for Japanese B encephalitis virus appeared in significant titers in 4 of 12 students inoculated with 4 to 6 cc of formalin-inactivated, angle-centrifuged, lyophilized mouse brain vaccine. Equivocal titers developed after vaccination in 3 additional members of this group, but intra-abdominal neutralization tests in very young mice gave negative results with these sera. The antibodies which appeared in significant titers persisted for at least 4 weeks. These data suggested that 4 cc of a vaccine, possessing a 50% immunogenic dose in mice of about 0.01 cc, was probably in the range of minimal dosage that would be required for human beings.

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Antibody Response of People of Different Ages to Two Doses of Uncentrifuged, Japanese B Encephalitis Vaccine.

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The development of neutralizing antibody in at least 4 of 12 students inoculated with 4 to 6 cc of an angle-centrifuged, lyophilized Japanese B encephalitis mouse brain vaccine was reported in the preceding communication.[1] Further experimental work on both Japanese B and St. Louis encephalitis mouse brain vaccines, indicated, however, that approximately 75% of the antigenic material may be lost by centrifugation.[2,3] While it was possible to obtain satisfactory lyophilization of these uncentrifuged vaccines on a laboratory scale, pilot tests by the methods employed commercially in 1943 yielded lyophilized products which were largely denatured and unsatisfactory.‡ For these reasons it was decided in 1944, that the Japanese B encephalitis vaccine that was to be prepared on a large scale for the armed forces was to consist of 10%, uncentrifuged mouse brain suspension in physiological salt solution in which the virus is inactivated by 0.2% formalin at 2 to 5°C, the final product to be stored and shipped in the fluid state in the cold. On the basis of both experimental studies in mice[2] and preliminary observations on the development of neutralizing antibodies[1,2] it was believed that for use in the face of an epidemic, the uncentrifuged, fluid vaccine should be administered in 2 doses, 2 cc each, 3 days apart. It was desirable, therefore, to know what the antibody response might be under these conditions, and the personnel of the commercial, biological houses, who were to produce this vaccine on a large scale, helped by giving their blood before and after vaccination in the manner described above.

The data on the preparation and assays of the vaccine for immunogenic potency in mice in this group of people are shown in Table I. The minimal immunogenic dose for mice determined, as indicated in Table I, was 0.0054 cc and 0.0056 cc in 2 separate tests, and almost identical values were obtained months later in several other laboratories, to which this vaccine was sent for use as a standard. Based on repeated assays on a number of vaccines prepared in this manner,[3] the minimal requirement for the vaccines prepared on a large scale was set at 0.01 cc for the 50% immunogenic dose. The serum specimens available for this study were obtained and prepared through the courtesy and cooperation of Dr. Betty Lee Hampil of Sharp and Dohme, Inc. and of Dr. Clara Nigg of the E. R. Squibb Co. The vaccine was given subcutaneously in the arm, 2 cc for the first dose and the same amount 3 days later for the second dose. The group at the Squibb Laboratories received the vaccine as is, without neutralization of the formalin, which was the procedure to be followed in the field. In order to

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* This work was done while the author was on active duty in the Army and on leave from the Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine.

† The work was carried out in the laboratories of the Rockefeller Institute for Animal and Plant Pathology at Princeton, N.J., and the indebtedness and gratitude of the author and the Commission are hereby expressed to Dr. H. S. Gasser and Dr. C. TenBroeck.

1 Sabin, A. B., and Duffy, C. E., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 123.

2 Sabin, A. B., Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. A. M. A.*, 1943, **122**, 477.

3 Sabin, A. B., unpublished tests.

‡ The Commission on Neurotropic Virus Diseases is indebted to Sharp and Dohme, Inc., for the pilot tests which they carried out, and to Dr. Robert Ward for his studies on this material in 1943.

TABLE I.

Data on Japanese B Encephalitis Mouse Brain Vaccine (Lot AB) Used on Human Volunteers.

Preparation—12/22/44—10% mouse brain suspension in physiological salt solution; uncentrifuged but filtered through absorbent cotton and gauze.
Lot A—370 cc—intracerebral titer = $10^{-9.3}$ per 0.03 cc.
Lot B—344 cc—intracerebral titer = $10^{-8.8}$ per 0.03 cc.
Formalin added to concentration of 0.2%.
Stored in ordinary refrigerator at about 4°C.
1/ 3/45—Lot A and Lot B pooled and phenylmercuric borate added to concentration of 1:50,000.
1/ 5/45—Tests for residual, infective Japanese B virus, safety tests in mice and guinea pigs, and assay for immunogenic potency carried out.
1/26/45 to 2/2/45—Used on human volunteers.

Assays for Immunogenic Potency in Mature Mice.

Mice— 18 to 20 g females.
Vaccination—Different dilutions of vaccine in volume of 1 cc injected intra-abdominally; 2 doses given 3 days apart.
Challenge— 1 week after first dose of vaccine; 0.3 cc of 10% virus suspension intra-abdominally.

Test No. and date	Intracerebral LD_{50} of virus in challenge dose given intra-abdominally	Mortality of unvaccinated controls	Mortality of mice vaccinated with indicated total dose					50% immunogenic dose ID_{50} cc
			0.1	0.03	0.01	0.003	0.001	
1. 1-12-45	$10^{8.8}$	20/20	1/20	1/10	3/9	5/10	10/10	.0054
2. 1-23-45	$10^{8.6}$	21/21	0/10	1/10	1/9	8/10	10/10	.0056

TABLE II.

Japanese B Encephalitis Virus Intracerebral Neutralization Test on Human Sera Before and Two Weeks After Vaccination with Lot AB.

Summary of test done on 27 February 1945.

Name and age	Serum specimen	Mortality at indicated final dilutions of virus					LD_{50} titer	Neutralization index
		10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
Normal rabbit serum heated at 56°C for 30 min	Control	—	5/5	5/5	5/5	0/5	8.5	—
Sus.—35	Prevaccination	5/5	5/5	5/5	5/5	—	8.5	1
	Postvaccination	5/5	4/5	2/5	0/5	—	6.7	63
Pan.—27	Prevac.	5/5	5/5	5/5	5/5	—	8.5	1
	Postvac.	4/5	1/5	0/5	0/5	—	5.5	1000
Fla.—20	Prevac.	5/5	5/5	5/5	5/5	—	8.5	1
	Postvac.	5/5	2/5	1/5	0/5	—	6.0	320
Dea.—18	Prevac.	5/5	5/5	5/5	4/5	—	8.4+?	1
	Postvac.	5/5	4/5	1/5	0/5	—	6.5	100
Hum.—19	Prevac.	5/5	5/5	5/5	3/5	—	8.2+?	2—?
	Postvac.	5/5	3/5	0/5	0/5	—	6.2	200

eliminate the pain resulting from the formalin in the vaccine the people at the Sharp and Dohme laboratories neutralized the formalin by the addition of $NaHSO_3$ just before inoculation. The sera, obtained before inoculation and 2 weeks after the first dose, were stored in the frozen state in an insulated box containing solid CO_2. The prevaccination and postvaccination specimens were always included in the same test, a typical example of which is shown in Table II. Frozen portions of the same lot of virus, and the same lot of heated, undiluted rabbit serum for the control mixtures, were used in

TABLE III.
Appearance of Neutralizing Antibodies for Japanese B Encephalitis Virus in Human Volunteers of Different Ages. Inoculated with Uncentrifuged, Fluid Mouse Brain Vaccine.

Two doses—2 cc each given 3 days apart			Intracerebral neutralization index*	
No.†	Name	Age yr	Before vaccine	2 wk after 1st dose of vaccine
1	Dea.	18	1	**80**
2	Hum.	19	2—?	**160**
3	Mas.	19	1	**1000**
4	Wil.	19	—1	**100**
5	Wal.	19	—1	1
6	Fla.	20	—1	**320**
7—	Har.	21	2—?	**250**
8	Bur.	22	—1	1—?
9—	Lon.	22	2—?	**50**
10—	Hil.	26	2—?	**400**
11	Dev.	26	—1	*10*
12—	New.	26	1	2—?
13	Pan.	27	—1	**800**
14	Mat.	27	1	**250**
15	McCon.	27	—1	8
16	Cra.	27	—1	1
17—	Ree.	28	—1	*10*
18	Pie.	30	2—?	*13*
19	Cla.	30	1	*13*
20—	Haw.	30	2—?	2—?
21—	Cat.	31	2—?	**63**
22—	Bis.	31	—1	6
23—	Sou.	31	1	1
24—	Hei.	34	—1	**800**
25	Sus.	35	—1	**50**
26	Fel.	36	—1	—1
27	Sho.	36	—1	1—?
28	Str.	39	—1	4—?
29	Glo.	40	4—?	**800**
30	Woo.	41	—1	1
31	Fit.	42	—1	2—?
32—	Lip.	47	—1	2—?
33—	Nig.	47	4—?	**630**
34	Ham.	48	1	2—?
35—	Jac.	56	1	1

* These neutralization indexes were calculated from the combined control LD_{50} titer of 8.4, determined from the results obtained with the control mixtures in all the tests.

The distinctly positive indexes are in boldface.

The equivocal indexes are in italic.

† The numbers followed by a dash indicate the people who received the vaccination without neutralization of the formalin; all the others received vaccine to which $NaHSO_3$ was added prior to inoculation.

all the tests. The serum-virus mixtures were incubated in a water bath at 37°C for 2 hours prior to intracerebral inoculation in the mice. The LD_{50} titers (the reciprocals of the log of the dilution) of the rabbit serum, control mixtures in the different tests were 8.8, 8.3, 8.0, 8.5, 7.7, and 8.7; that these variations are inherent in the test is evident from the fact that in a test in which the control mixture yields a titer of 7.7, the titer of the virus in a mixture with prevaccination serum may be 8.5, and that material from the same ampule of virus mixed with the same lot of rabbit serum which yielded the titer of 7.7 gave a titer of 8.7 in a test carried out 2 weeks later. For these reasons, the neutralization indexes shown in Table III were all calculated from a combined, control LD_{50} titer (8.4) which was determined by the method of Reed and Muench from the sum of the results obtained with the control mixtures in the 6 separate tests.

The results of all the tests are shown in Table III, arranged according to the age of the vaccinated people. The complete absence of the slightest antiviral effect in any of the freshly frozen sera obtained before vaccination from this group of 35 people residing in the northeastern part of the U.S.A. is especially noteworthy, in view of the very high incidence of antibodies encountered among adults where Japanese B encephalitis is endemic[4] as well as occasionally among residents in the U.S.A.[2] Those who developed antibodies following vaccination exhibited neutralization indexes of 50 to 1000, and only 4 had indexes of 10 to 13, the equivocal nature of which was discussed in the preceding communication.[1] Inspection of the results in Table III suggested the possibility that the younger adults may perhaps develop antibody more regularly than the older people. From the summary presented in Table IV, it appears that 52% of those 18 to 35 years of age developed antibodies (68%, if the equivocal indexes are included) as compared with 20% of those who were 36 to 56 years old. Although this data is not conclusive, it warrants further observations on the influence of the age of human beings on the development of antibodies following vaccination. The results summarized in Table IV also indicate that neutralization of the formalin with $NaHSO_3$, immediately before

4 Sabin, A. B., *J. A. M. A.*, 1947, **133**, 281.

TABLE IV.
Influence of Age of Vaccines and Neutralization of Formalin Prior to Inoculation, on Incidence of Neutralizing Antibodies.

Group	Total No.	Positive No.	Equivocal No.	Negative No.	Positive %	Positive + equivocal %
All ages	35	15	4	16	43	54
18-35 yr	25	13	4	8	52	68
36-56 "	10	2	0	8	20	20
Formalin not neutralized	13	6	1	6	46	54
Formalin neutralized with $NaHSO_3$ prior to inoculation	22	9	3	10	41	55

inoculation. has no effect on the development of antibodies.

The results obtained in this study indicated that approximately 50% of the people may develop neutralizing antibodies following injection of a total dose of 4 cc (2 doses of 2 cc, 3 days apart) of a Japanese B encephalitis vaccine possessing a 50% immunogenic dose of about 0.005 cc as determined by mouse assay. Thus far, it has not been possible to prepare vaccines of significantly greater antigenic potency, and the administration of larger amounts of mouse brain vaccine has been regarded as undesirable. Although resistance to infection may also be present among the 50% of vaccinated people who fail to develop antibodies, just as it can be demonstrated in mice inoculated with small amounts of vaccine,[2] this dosage of vaccine was selected for human beings because it was expedient rather than optimum.

Summary. Thirty-five people, aged 18 to 56, whose prevaccination sera were without any antiviral effect on Japanese B encephalitis virus, received 2 doses, 2 cc each, 3 days apart, of a fluid, uncentrifuged, formalinized vaccine with a 50% immunogenic dose for mice of about 0.005 cc. Two weeks after the first dose of vaccine, 43% had antibodies with intracerebral neutralization indexes of 50 to 1,000, and 54% were positive if the equivocal indexes of 10 or more are included. The incidence of antibody development appeared to be higher among the younger adults (18 to 35 years) than among the older ones (36 to 56 years) but this requires confirmation. Neutralization of the formalin with $NaHSO_3$ immediately before injection of the vaccine had no effect on the development of antibodies.

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Neutralizing and Complement-Fixing Antibodies for Japanese B Encephalitis Virus in Vaccinated U. S. Personnel in Japan.

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The explosive character and unpredictability of epidemics of Japanese B encephalitis, the lack of immunity of the occupation forces

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TABLE I.

Serological Response of U. S. Adult Personnel in Japan to 3 Doses of 1 cc Each of Japanese B Encephalitis Mouse Brain Vaccine

No.	Subject and age	Date of arrival in Japan	Neutralizing antibodies: Before vaccine	Neutralizing antibodies: 10 days after 2nd dose	Neutralizing antibodies: 10 days after 3rd dose	Complement-fixing antibodies: Before vaccine	Complement-fixing antibodies: 10 days after 2nd dose	Complement-fixing antibodies: 10 days after 3rd dose
1	B. (19)	10-45	13—?; 40?*	**4000+**	**4000+**	0	32(2)	16(2)†
2	McN. (30)	10-45	20—?; 1	**320+?; 8000**	**4000+**	0	32(2)	16(2)
3	T. (31)	9-45	2	*32*	**4000+**	0	2(2)	4(2)
4	S. (33)	6-46	3	**320+**	**400**	0	0	0
5	Br., J. (20)	10-45	5—?; 1	*20; 16*	**320**	0	4(4)	4(4)
6	K. (25)	10-45	4	*32*	*40*	0	0	0
7	P. (26)	5-46	3	*20*	**50**	0	0(2)	2(2)
8	C. (23)	11-45	4	4	**200**	0	2	4(2)
9	Po. (28)	4-46	5	6	**4000+**	0	0	2(2)
10	L. (20)	9-45	25—?; 8	20—?; 8	**800**	0	0(2)	0(2)
11	H. (20)	11-45	5—?	5—?	*32—?; 25*	0	0	4(?)
12	Br., C.	?-?	20—?; —2	4—?	5—?	0	0(4)	4(4)
13	Ho. (22)	10-45	20—?; 6	8—?	8—?	0	0	0
14	Ca. (20)	11-45	4—?	8—?	5—?	0	0	0
15	E. (20)	3-46	5—?	5—?	8—?	0	0	0
16	Hol. (19)	4-46	8—?	5—?	8—?	0	0	0
17	McG. (19)	4-46	4	4	4—?	0	0	0
18	En. (30)	9-45	13—?; 4	4	5—?	0	0	0
19	Ke. (35)	4-46	6	8	8—?	0	0	0
20	Pow. (28)	5-46	1	3	8—?	0	0	0
21	M. (40)	4-46	—3	13—?	6	0	0	0
22	G. (61)	6-46	8—?	5—?	8—?	0(2)	2(2)	0(2)
23	A. (previous vaccine)	10-45	—1	**80**	*32*	0	0	0
24	Le. (23)	5-46	**320+; 500**	**320+**	**4000+**	0	2(2)	0

The neutralization indexes in boldface are regarded as positive, and those in italic as equivocal.

* Where 2 neutralization indexes are given, the second one represents the result obtained on repetition of the test.

† 16(2) = complement fixation with the Japanese B virus antigen in an original serum dilution of 1:16 and with the mouse brain component (*i.e.*, the Western Equine or normal mouse brain antigens) in a dilution of 1:2; the reactions with antigens other than Japanese B are given in parenthesis. These titers should be multiplied by 4 to make them comparable to the final serum dilution titers reported by others.

in Japan, and the administrative difficulties experienced in vaccinating large numbers of people during the outbreak on Okinawa in 1945, were factors in the decision to vaccinate the occupation forces in advance of the season when epidemics might occur. In view of the fact that the antigenic potency of the best Japanese B encephalitis vaccines that can be made at this time is limited,[1] and that it was considered desirable to keep the amount of mouse brain to be inoculated down to a minimum, it was decided to alter the dosage, from 2 doses of 2 cc each, 3 to 5 days apart, which was selected for use during an epidemic, to 2 doses of 1 cc each 4 to 7 days apart, to be followed by a booster dose of 1 cc one month later, or prior to 15 June on Okinawa and 15 July in Japan, or earlier if an epidemic appeared. The purpose of the present study was to determine the serological pattern, both as regards neutralizing and complement-fixing antibodies, in U.S. personnel in Japan receiving, accord-

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[1] Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 127.

ing to the schedule just outlined, the commercially prepared Japanese B encephalitis mouse brain vaccines produced in the U.S.A. The results were especially needed to provide a base-line for interpreting the serologic picture that might be found in vaccinated individuals with illnesses suggesting nonbacterial infections of the nervous system. It was furthermore desirable to correlate the data to be obtained with the antigenic potency of the commercial vaccines as assayed in mice during the period of their use, and with the results of previous studies in which vaccines of known potency were used in different dosage.

The studies were carried out on 24 Americans in Tokyo, whose arrival in Japan varied from late September, 1945 to early June, 1946. All but 2 were under 40 years of age. All but 2 had not previously been in any country where Japanese B encephalitis is known to occur; the 2 possible exceptions (No. 3 and 18 in Table I) had been in the Philippines from March to September, 1945. All but one (No. 23) had not previously received Japanese B encephalitis vaccine. The first dose of vaccine (1 cc) was given between 11 and 14, June, 1946; the second dose (1 cc), 4 to 5 days later; and the third dose (1 cc), 30 to 31 days after the first. The inoculations were given at an army dispensary where many others received the same lots of vaccine. Two lots of vaccine were used, samples of which were sent, refrigerated, to the Division of Virus and Rickettsial Diseases of the Army Medical School in Washington, D.C. for assay, and we are indebted to Doctors Joel Warren and Joseph E. Smadel for the results. The lot used for the first dose was prepared 30 November, 1945, (the one-year expiration date was 11-30-46) and upon assay in mice on 30 August, 1946, it yielded a 50% immunogenic dose (ID_{50}) of 0.028 cc. Since before being released for use it must have passed the minimal potency requirement of an ID_{50} of 0.01 cc in tests by the National Institute of Health, this lot of vaccine lost at least about ⅔ of its original potency. Whether this loss is the result of improper refrigeration, too small or too large an amount of residual formalin, or of other factors is not known. The lot of vaccine used for the 2nd and 3rd doses was prepared by another commercial company on 8 August, 1945, and upon assay in mice on 4 September, 1946, it yielded an ID_{50} of 0.0094 cc, and thus still fulfilled the minimal requirements.

Blood was obtained just before vaccination, 10 days after the 2nd dose and 10 days after the 3rd dose. The sera were all stored in the frozen state in an insulated box containing solid CO_2, and the pre- and postvaccination specimens were always tested simultaneously. The intracerebral neutralization tests were carried out and the indexes calculated from the combined, control LD_{50} titer in the manner described in the preceding communication.[1] The control LD_{50} titers of different portions of the same lot of virus used in the 6 separate tests were 8.5, 7.5, 8.5, 7.8, 8.0 and 8.2 (the reciprocals of the log of the dilution) and the combined titer of 8.1 was used for calculating all the neutralization indexes shown in Table I, with the exception of those obtained on repetition when different lots of virus were used. The complement fixation tests were carried out essentially according to the method of Casals and Palacios.[2] The sera were mixed with equal parts of physiological salt solution and heated at 60°C for 20 minutes just before the test; the undiluted sera were not tested because the results obtained with them cannot be regarded as significant. The antigens were all prepared from mouse brains infected with the Japanese B, St. Louis, or Western equine encephalitis viruses, or normal mouse brains for control, without freezing and thawing, by centrifugation at 18,000 r.p.m. on the International Centrifuge angle-head attachment (refrigerated by dry ice) for 60 minutes or longer, if necessary for the removal of anticomplementary material demonstrable by incubation overnight in the refrigerator. The preparations were used undiluted and had at least 4 to 8 units of antigen in the 0.25 cc amounts used in the test. Tests in which the amount of complement used turned out

2 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

TABLE II.
Types of Complement-Fixing Reactions with Various Antigens Exhibited by Sera of Americans in Japan Inoculated with Japanese B Encephalitis Mouse Brain Vaccine.

		Complement-fixation in mixtures with indicated antigens						
Name	Specimen*	Japanese B Serum 1: 2 4 8 16 32 64 128	St. Louis Serum 1: 2 4 8 16	WEE Serum 1: 2 4 8	NMB Serum 1: 2 4 8	Saline Serum 1: 2	C-F titer for Jap B	C-F titer for WEE or NMB
	I	0 0 0 0	0 0	0 0	0	0	0	0
McN.	II	4 4 4 4 3 ±	4 ± 0 0	2 0 0	2 0 0	0	1:32	1:2
	III	4 4 4 2 0 0 0	3 ± 0 0	1 0 0	4 0 0	0	1:16	1:2
	I	0 0 0 0	0 0	0 0	0	0	0	0
Bag.	II	4 4 4 4 3 1 0	4 2 1 ±	2 0 0	1 0 0	0	1:32	1:2
	III	4 4 4 3 ± 0 0	4 2 ± 0	2 0 0	2 0 0	0	1:16	1:2
	I	0 0 0 0	0 0	0 0	0	0	0	0
Har.	II	1 0 0 0	1 0	0 0	±	0	0	0
	III	4 3 ± 0	0 0	0 0	1	0	1:4	0?
Bro.,	I	0 0 0 0	0 0	0 0	0	0	0	0
J.C.	II	4 3 1 ±	4 3	4 3	4	0	1:4	1:4
	III	4 3 1 0	4 3	4 3	4	0	1:4	1:4
Bro.,	I	0 0 0 0	0 0	0 0		0	0	0
C.A.	II	1 ± 0 0	3 3	2 2		0	0	1:4
	III	3 2 0 0	3 2	2 1		0	1:4	1:2
	I	0 0 0 0 0	0 0	0 0	0	0	0	0
Sch.	II	0 0 0 0 0	0 0	0 0	0	0	0	0
	III	0 0 0 0 0	0 0	0 0	0	0	0	0

* I = serum before vaccine; II = 10 days after 2nd dose; III = 10 days after 3rd dose.

C-F = complement-fixing antibody; WEE = Western Equine encephalitis virus; NMB = normal mouse brain antigen; Saline = mixture with physiological salt solution instead of antigen to check on anticomplimentary properties of the serum.

Complete fixation is recorded as 4; different degrees of partial fixation as 3, 2, or 1; questionable trace as ±; no fixation, or complete hemolysis, as 0. The original dilution of serum giving 2 plus (approximately 50%) fixation represents the titer.

to be less than 1.7 or more than 2.5 units, as determined by simultaneous titration in the presence of the antigens were regarded as unreliable, and were repeated. The various types of reaction with the different antigens, exhibited by the sera obtained after vaccination, are illustrated by the results of a few tests in Table II. The results of all the complement fixation and neutralization tests are shown in Table I.

Only one of the 24 people had neutralizing antibodies for the Japanese B encephalitis virus before vaccination. This man was born in New York City where he lived all his life with the exception of approximately one year each in Arizona, California and Kansas. Prior to coming to Tokyo in May, 1946 he spent 2 months each in Saipan and Hawaii. His serum failed to neutralize the virus of St. Louis encephalitis. The occurrence of neutralizing antibodies for the Japanese B virus in one of 30 sera received from New Haven was reported in 1938 by Japanese investigators,[3] and also in 1943 among a varying number of medical students living in Cincinnati, Ohio.[4] Since these antibodies have been found in sera which do not neutralize the St. Louis encephalitis virus, they cannot be attributed to exposure to that virus and the slight antigenic relationship between it and the Japanese B virus. It is noteworthy that the one man with neutralizing antibodies in the present group had no complement-fixing antibodies for the Japanese B virus and that none appeared after vaccination. This is in contrast to an observation made by Dr. Hammon as well as ourselves, that complement-fixing antibodies appear rapidly and regularly after vaccination of Japanese natives possessing neutralizing antibodies as a result of inapparent infection with the virus.

[3] Takaki, I., Kudo, M., Kawakita, Y., and Tanaka, J., *Tokyo Izi Sinsi*, 1938, **62**, 716. (In Japanese; reference and translation provided by Dr. Y. Kawakita).

[4] Sabin, A. B., Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. A. M. A.*, 1943, **122**, 477.

TABLE III.

Neutralizing Antibodies for Japanese B Encephalitis Virus in Two Groups of People of Similar Age Following Vaccination with Different Amounts and Preparations of Different Potency.

Group	ID_{50} of vaccine by mouse assay cc	No. of persons in group	Dosage cc	Incidence of neutralizing antibodies at indicated times: Time after vaccine	Positive %	Positive + equivocal %
American adults 18-35 years in U.S.A. (reported by Sabin[1])	0.0055	25	1st dose—2 2nd dose—2 3 days later	14 days after 1st dose	52	68
American adults 19-35 years in Japan Present study	0.028 (1st dose) 0.0094 (2nd & 3rd doses)	20	1st dose—1 2nd dose—1 4-5 days later 3rd dose—1 1 month after 1st	14-15 days after 1st dose 10 days after 3rd dose	15 45	35 55

In order to permit comparison of the results obtained in the present study (Table I) with those reported in the preceding communication[1] the analysis of the data will be limited to the first 20 men, aged 19 to 35, who had no antibody or previous history of receiving Japanese B encephalitis vaccine prior to the present series of inoculations. Ten days after the second dose of 1 cc (*i.e.* 14 to 15 days after the first), 3 of the 20 (15%) developed significant titers of neutralizing antibody (indexes of 320, 4000+, and 8000) and 4 others (20%) exhibited equivocal titers of 20 to 32. Ten days after the 3rd dose (booster of 1 cc given one month after first dose), 3 of the 4 with equivocal titers after the 2nd dose became positive with indexes of 50, 320, and 4000+, and 3 additional individuals, who were previously negative, became positive with indexes of 200, 800, and 4000+. Thus, following the 3rd dose of vaccine, 9 of the 20, or 45%, had demonstrable neutralizing antibodies of significant titer and 2 additional ones (10%) exhibited equivocal titers. When these results are compared (Table III) with those obtained in 25 adults of similar age in the U.S.A. who received only 2 doses of 2 cc of a vaccine of greater potency, as determined by mouse assay, it is evident that:

(a) The 2 doses of 2 cc of the better vaccine produced better results (52% positive) than the 2 doses of 1 cc (15% positive) of the poorer vaccines.

(b) With the poorer commercial vaccines available in the field, a total dose of 3 cc (given in 2 doses of 1 cc, 4 to 5 days apart, followed by a booster of 1 cc one month later) gave practically the same results (45% positive) as a vaccine of 2 to 5 times greater potency in a total dose of 4 cc (given in 2 doses of 2 cc, 3 days apart).

The complement-fixing antibodies which were found following vaccination were of 2 kinds: one, reacting with the various virus antigens as well as with normal mouse brain in serum dilutions of 1:2 to 1:4, appeared in 7 of the 20 men (35%) after the 2nd dose and in 9 (45%) after the 3rd dose; the other, apparently specific for the Japanese B encephalitis virus and demonstrable in serum dilutions of 1:16 to 1:32, appeared in only 2 individuals (10%), and in both instances the titer was higher after the 2nd dose (1:32) than after the 3rd (1:16). In a third man ("Har." in Table II, No. 11 in Table I), with the serum obtained after the 3rd dose of vaccine, there was definite fixation in a dilution of 1:4 only with the Japanese B antigen, and, although entirely absent with the St. Louis and Western equine antigens, there was very slight (one plus) fixation with the normal mouse brain antigen. However, since the nonspecific reaction (*i.e.* against the mouse brain component) may yield, within the range of a

4-fold dilution, different titers with the different antigens (see No. 10 and 12 in Table I and Bro., C. A. in Table II), it seemed most probable that this seemingly specific fixation with the Japanese B virus antigen was actually nonspecific. For if this were interpreted as development of specific complement-fixing antibody, one would have to conclude in another case (Bro., C. A. in Table II) that the same vaccine gave rise to complement-fixing antibodies for the Western equine and St. Louis viruses, but not for the Japanese B virus. We concluded from these data that in people who had been inoculated with mouse brain vaccine, complement-fixation titers not greater than 1:4 of original serum dilution obtained with any mouse brain antigen, could not be regarded as specific (except for the mouse brain component) even when the reaction was positive with only one of a series of several antigens.

Summary. Commercial Japanese B encephalitis mouse brain vaccine was administered to the occupation forces in Japan in 1946 by the triple-dose method, and the antibody response was studied in a group of men who received the first 2 doses of 1 cc each 4 to 5 days apart and the 3rd dose of 1 cc one month after the first dose. The vaccine used for the first dose had a 50% immunogenic dose (ID_{50}) of 0.028 cc by mouse assay, which is considerably less than the minimal required potency of 0.01 cc, and the lot used for the other 2 doses just fulfilled the minimal requirements with an ID_{50} of 0.0094 cc. The results in 20 Americans, aged 19 to 35, who were stationed in Tokyo and were without antibody or previous history of having received this vaccine, were as follows:

(a) 15% had neutralizing antibodies 10 days after the 2nd dose, and 45% 10 days after the 3rd dose.

(b) Only 2 men (10%) developed specific complement-fixing antibodies for the Japanese B virus, which appeared after the 2nd dose of vaccine.

(c) Complement-fixing antibodies for the mouse brain component of the vaccine appeared in original serum dilutions of 1:2 to 1:4 in 35% after the 2nd dose and in 45% after the 3rd dose of vaccine.

The antibody response in this group of Americans in Japan, vaccinated by the triple-dose method with commercial preparations, which either just fulfilled or were below the ID_{50} of 0.01 cc which is the minimal requirement of potency, was about the same after the 3rd dose as that previously obtained after the 2nd dose in a group of 25 Americans, of similar age in the U.S.A., who received 2 doses of 2 cc each, 3 days apart, of a freshly prepared vaccine with an ID_{50} of 0.0055 cc.

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Serological Response of Japanese Children and Old People to Japanese B Encephalitis Mouse Brain Vaccine.

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A field trial to test the value of Japanese B encephalitis vaccine for the protection of children and old people living in the endemic

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TABLE I.

Serologic Response of Japanese Natives in Japan to Japanese B Encephalitis Mouse Brain Vaccine.

A. Influence of Potency of Vaccine as Determined by Mouse Assay.
B. Influence of Age of Vaccinated People.
C. Influence of Previous Inapparent Infection on Appearance of Complement-Fixing Antibodies.

Lot of vaccine and potency ID_{50}	Individuals vaccinated			Neutralization index*			Complement-fixing titer†		
	No.	Name	Age yr	Before vaccine	10 days after 2nd dose	10 days after 3rd dose	Before vaccine	10 days after 2nd dose	10 days after 3rd dose
A	1	Sak.	5	3	320	3200+	0; 0	4(2)	4(?)
0.013 cc	2	Shi.	3	3	160	3200+	0	0; 2(2)	8(4); 16(4)
	3	Yam.	5	4	63	3200+	0	0	2(2)
	4	Yag.	4	6	25	500	0(2)	2(2)	4(2)
	5	Mor.	3	3	3	63	0	0	8(4)
	6	Han.	5	—2	2	160	0	2(?)	4(4)
	7	Kuy.	4	3	2	400	0	0	4(?)
	8	Nis.	4	2	2	2500	0	0	8(2)
	9	Hir.	3	4	2	2000	0; 0	0	8(4)
	10	Has.	5	—	—2	3200+	—	0	8(2); 8(0)
B	1	Ino.	5	1	3	500	0	0	0
0.077 "	2	Kaw., M.	5	3	6	20—?	0	0	AC, <4(4)
	3	Nak., T.	5	3	6	16—?	0	0	2(?)
	4	Kaw., T.	5	2	—2	16—?	0	0	0
	5	Kat.	5	2	3	5	0	0	0
	6	Ish.	5	1	2	6	0	2(2)	4(2)
	7	Kai.	4	—2	—2	6	0	0	8(4); 2(2)
	8	Kis.	4	2	3	4	0	0	0
	9	Nak., R.	4	3	—3	3	0	0; 2(2)	0; 2(2)
	10	Yok.	3	3	—2	3	0; 0	0; 0(2)	0
	1	Kaw., Ts.	65	2	2	4	0	0	0
	2	‡Aos., C.	70	1	6	4	0	0	0
A	3	Ish., J.	70	3	3	25; 5	0	0	0
	4	Sug.	61	4	1	10; —2	0	0	0
	5	Kob.	76	1	—3	3	0	0	0
	6	Yas.	71	—2	—2	3	—	0	0
	7	Iba.	63	—2	2	4	0	0	0
	8	Non.	63	—3	4	3	0	0(2)	2(2)
	9	Tsu.	63	3	2	4	0	0	0(2)
	1	Yok.	73	320+	—	—	2(2)	16(2)	16(2)
	2	Sui.	62	2500	—	—	2(4); 2(2)	8(4); 8(2)	8(2)
	3	Kan.	80	3200+	—	—	0	16	—

B	4	Tam.	68	250	—	—	0	8	4
	5	Kaw.	78	320+	—	—	0	8	8
	6	Kur.	67	320+	—	—	0	16	4
	7	Kaw., J.	64	320+	320+	2500	0	32	8
	8	Kom.	73	250	—	—	2	32	8
	9	Kaw., S.	68	320+	—	—	2	64	32
	10	†Aos., K.	64	320+	—	—	4	16	8

* The neutralization indexes not preceded by a minus sign represent the ratio of the combined, control LD_{50} titer to that of the serum; when the titer of the serum was greater than that of the control, the ratio was reversed and the result recorded as a negative quantity.

† The complement-fixing titer represents the original dilution of serum yielding 2 plus (approximately 50%) fixation; the values given in parentheses represent the titers with normal mouse brain or Western Equine encephalitis antigen. When a titer is not followed by another in parentheses, it means that complement-fixation occurred only with the Japanese B encephalitis antigen. 0 = no fixation in the lowest dilution which was 1:2. Where 2 sets of values are given separated by a semicolon, they represent the results obtained on repetition of the test.

‡ Missed 2nd dose of vaccine; booster dose given 1 month after 1st.

region of Okayama, Japan, was carried out during the summer of 1946 in association with Professors K. Kitayama and E. Hamamoto, Doctors K. Hiraki and S. Shimomura and their associates on the medical faculty of the University of Okayama, and Professor M. Kitaoka of the Government Institute for Infectious Diseases, Tokyo Imperial University. In view of the limited amount of vaccine which was available for this investigation, our Japanese associates believed that the study should be limited to children 3 to 5 years of age and to old people over 60, because the case fatality rate among the latter can be 70 to 90%. Accordingly, approximately 19,000 children and 2,000 old people were inoculated with the same commercial vaccine, which was prepared in the U.S.A. and used for all the occupation forces, and by the same triple-dose method in advance of the season when epidemics of encephalitis usually appear. The children and the old people received the same amounts of vaccine, the first 2 doses of 1 cc each being given 6 days apart and the 3rd dose of 1 cc, one month after the first dose. The present study was undertaken not only to determine the antibody response of a small group of these people to vaccination but also to provide a base-line for interpreting the serologic pattern which might be encountered subsequently should any of these vaccinated people develop illnesses requiring laboratory investigation of the etiological relationship of the virus of Japanese B encephalitis.

Ten children and 10 old people living in Tsuyama City and a similar number living in a rural area (Kume-son and Miho-cho) were selected for these studies. Blood was obtained immediately before the first dose of

‖ We wish to express our indebtedness to Brig. Gen. S. Bayne-Jones, at the time deputy chief of the Preventive Medicine Service, Office of the Surgeon General, U. S. Army, Brig. Gen. J. I. Martin, U. S. A., Chief Surgeon of the U. S. Army Forces in the Pacific, Col. Crawford F. Sams, M.C., chief of the Public Health and Welfare Section of the Supreme Command for the Allied Powers, and Lt. Col. W. D. Tigertt, M.C., commanding officer of the 406th Medical General Laboratory, for making possible the work of this commission.

vaccine, 10 days after the 2nd dose, and 10 days after the 3rd dose. We are indebted to Capt. J. S. McKinney, M.C. and Lt. W. A. Scantland, M.C., who along with other medical officers worked with the Commission on this project, for obtaining the blood specimens on these people and shipping them in refrigerated containers to the Tokyo laboratory. The sera were then stored in the frozen state in an insulated box containing solid CO_2. First, the prevaccination specimens were all tested for neutralizing antibodies. All of the children and 9 of the 20 old people had no demonstrable antibodies. The 2 postvaccination specimens on each individual who had no antibodies were then tested together. The prevaccination and postvaccination specimens were tested simultaneously for complement-fixing antibodies. The neutralization tests were carried out as before with the undiluted, unheated sera against at least 3 or 4 dilutions of the Nakayama strain of Japanese B encephalitis virus; frozen portions of the same lot of virus were used in all the tests and the neutralization indexes, reported in Table I, were calculated from the combined, control LD_{50} titer of $10^{-8.0}$, the individual, control, intracerebral titers having varied from $10^{-7.4}$ to $10^{-8.6}$. The complement-fixation tests were carried out as described before[1] with undiluted antigens and various dilutions (1:2 or higher) of the sera which were routinely heated at 60°C for 20 minutes; a few of the sera, treated in this manner, remained anticomplementary and had to be heated at 65°C for 20 minutes. Four mouse brain antigens were used, prepared from brains infected with the Japanese B, St. Louis or Western equine encephalitis viruses, and from normal mouse brain. One lot of vaccine (A) was used for all 3 doses in the urban group of this series, and another lot (B), for all 3 doses in the rural group. Aliquot portions of both lots were sent refrigerated to the Division of Virus and Rickettsial Diseases of the Army Medical School at Washington, D.C., and we are indebted to Doctors Joel Warren and Joseph E. Smadel for the results of the mouse assays. Lot A had a 50% immunogenic dose (ID_{50}) of 0.013 cc which is close to the minimal requirement of 0.01 cc. while Lot B (prepared at about the same time by another commercial company) had an ID_{50} of 0.077 cc or 1/6 of the potency of Lot A.

The results, which are all summarized in Table I, clearly show the influence of the potency of the vaccine, as determined by mouse assay, the age of the vaccinated people, and of previous inapparent infection on the antibody response. In comparing the 2 groups of children, one finds that all 10 of those receiving the better vaccine had developed good titers of neutralizing antibodies, while only 1 of 10 receiving the poorer vaccine, with 1/6 the antigenic potency, developed a distinctly positive neutralization index; furthermore, after the 2nd dose none of the children inoculated with vaccine B had any antibodies while 3 of the 10 receiving vaccine A had distinctly positive indexes. The influence of age is clearly evident when one compares the 10 children and 7 old people, without antibodies prior to inoculation, from the same community who received the same vaccine A at the same time; not one of the old people developed antibodies while all the children did. It was pointed out in the preceding communication[1] that complement-fixing antibodies for the mouse brain component of the vaccine (as is evident from the reactions with the normal mouse brain and Western equine encephalitis virus antigens) as well as for the Japanese B encephalitis virus developed following inoculation. It is noteworthy, however, that all 10 children, inoculated with vaccine A had complement-fixing antibodies, and that the titers were higher with the Japanese B virus antigen in 8 of them with a 4-fold difference in titer in 5. This is to be contrasted with the absence of any evidence of specific response among the children inoculated with vaccine B, and of any significant response of any kind in the old people who were without neutralizing antibodies prior to vaccination. Quite a different response was found in the old people

[1] Ginder, D. R., Matumoto, M., Schlesinger, R. W., and Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 130.

who had neutralizing antibodies prior to vaccination apparently due to previous inapparent infection with the virus of Japanese B encephalitis. All of these people showed a prompt appearance or very significant rise in complement-fixing antibodies only for the Japanese B virus antigen; this was most marked after the 2nd dose of vaccine and although it was still present about a month later (10 days after the 3rd dose) the titers were 2- to 4-fold less in 6 of the individuals. That this booster effect probably may result from a single dose of vaccine is suggested by the data on the last individual listed in Table I who did not receive the 2nd dose of vaccine. It is, furthermore, apparent that this booster effect may be produced by a vaccine of very poor antigenic potency, since 7 of the 10 old people exhibiting this effect were inoculated with vaccine B. It is also noteworthy, that none of these sera exhibited any specific group reaction with the St. Louis virus, even when the titers with the Japanese B virus were as high as 1:32 to 1:64.

Discussion. In view of the fact that this study was carried out in a region of Japan in which Japanese B encephalitis is known to be endemic, the question naturally arises to what extent the antibody response which was observed might have been the result of spontaneous infection during the period of investigation (14 June to 24 July) rather than due to vaccination. The following facts are against the possibility of spontaneous infection:

(a) Careful field, clinical, and laboratory investigations carried out as part of the vaccination program revealed no cases of Japanese B encephalitis in these areas during the summer and autumn of 1946.

(b) Concurrent studies revealed that during nonepidemic years, while the virus continues to be disseminated among certain domestic animals, the great majority, if not all, of the children born after the last epidemic are without antibodies.[2]

(c) Following inoculation with the vaccine of adequate potency, the neutralizing antibodies appeared in all the children but in none of the old people living in the same community.

(d) The final serum specimens for this study were obtained several weeks before the date when outbreaks usually reach their peak in this part of Japan.

The influence of the potency of the vaccine, as determined by mouse assay, on the antibody response in human beings, which was only suggested in the previous studies appears to be established by the results of the present investigation. That older people may not respond as well to the same dosage of vaccine was first suspected in a study carried out in the U.S.A.,[3] but the results of the present investigation, which are more striking because of the extremes of age and the seemingly all or none character of the response, add greater weight to this observation. The extent to which the larger amount of vaccine per unit of body weight might have been responsible for the better response of the children is not clear. It may be worth noting, however, that in the concurrent study on the response of U.S. personnel, aged 19 to 35 years, vaccinated by the same triple-dose method with preparations of equivalent or poorer potency, 45% had antibodies after the 3rd dose, in contrast to the completely negative results in the small group of old people; here the difference in the amount of vaccine per unit of body weight, if anything, favored the Japanese old people, who on the average probably weighed only about half as much as the group of American adults. It is interesting to speculate whether the poorer immunogenic response of the old people may be related to the high case fatality rate of the disease among them. The rapid and regular appearance of complement-fixing antibodies for the Japanese B virus following vaccination of the old people who exhibited evidence of previous inapparent infection, is in agreement with an observation made by Hammon[4] earlier in 1946 on a small group of Japanese adults. Further investigations are

2 Sabin, A. B., Ginder, D. R., and Matumoto, M., in press.

3 Sabin, A. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 127.

4 Hammon, W. McD., personal communication.

indicated to determine whether this may provide a method for distinguishing between neutralizing antibodies in people and animals which are the result of exposure to the virus, and "antibodies" which are occasionally found in animals and human beings under conditions where exposure to the specific virus is most unlikely. As an example, may be cited the American, reported in the preceding communication,[1] whose serum, prior to vaccination, neutralized the Japanese B encephalitis virus, but who did not develop complement-fixing antibodies following vaccination.

The results of the present investigation emphasize the importance of controlling the antigenic potency of Japanese B encephalitis vaccine by proper assay in mice, and indicate that the minimal requirement of 0.01 cc for the ID_{50} should be retained until vaccines of greater potency can be prepared. It would also appear that some vaccines lose their potency more quickly than others (this seemed to be especially true for the vaccines prepared by one commercial company), and checks on the residual amounts of formalin and study of certain other factors which may be involved are indicated to insure minimal acceptable potency for the preparations finally used in the field.

Summary. Japanese children, 3 to 5 years old, and people, over 60 years of age, living in the endemic region of Okayama, Japan, were given commercial Japanese B encephalitis mouse brain vaccine—2 doses of 1 cc each, 6 days apart, and a 3rd dose of 1 cc one month after the first dose in advance of the season when outbreaks of the disease may be expected. None of the children had antibodies before vaccination, and of the 10, who were inoculated with a vaccine having a 50% immunogenic dose (ID_{50}) of 0.013 cc by mouse assay, all had neutralizing antibodies 10 days after the 3rd dose, while only 1 of the 10, who received a vaccine with an ID_{50} of 0.077 cc, developed a significant neutralization index. However, in a group of 7 old people, without antibodies before inoculation, who received the same dosage of the better vaccine (ID_{50} of 0.013 cc) none developed neutralizing or complement-fixing antibodies. Complement-fixing antibodies for the mouse brain component appeared as well as for the Japanese B virus contained in the vaccine, but in 5 of the 10 children, inoculated with the better vaccine, the titers were significantly higher with the Japanese B antigen. Complement-fixing antibodies, specific for the Japanese B virus, appeared rapidly in all of the 10 old people who had neutralizing antibodies (evidence of previous inapparent infection) prior to inoculation, but in none of the 9 old people who were without such antibodies.

15891 P

Ragweed Reagins in Nasal Secretion.*

MAX SAMTER AND ELMER L. BECKER. (Introduced by W. H. Welker.)

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Although nasal secretion has been suspected by numerous observers to be an important factor in the mechanism of respiratory allergies, its mode of action is as yet unknown. The present study attempts to determine whether nasal secretion contains reagins in ragweed sensitive patients.

Experimental Procedure. Four normal subjects and 10 ragweed sensitive patients with marked skin reactivity and demonstrable circulating reagins were selected for the experi-

* This study was supported by a grant from the Asthmatic Children's Aid Society, Chicago, Ill.

ments, 2 months after the end of the ragweed season.

Nasal secretion was obtained by insertion into each nostril of cotton plugs saturated with 10% sodium chloride solution; satisfactory secretory response was usually obtained without appreciable irritation within 10 minutes. The material obtained was transferred immediately into 2 micro-Seitz filters each of 2.0 ml capacity, and pressed through the filters by centrifuging for 40 minutes at moderate speed. The filtrates were clear, slightly viscous and represented approximately one-fifth of the original volumes.

The cell free liquid thus prepared was used for passive transfer studies. 0.1 ml was injected into each of 4 sites (left arm) of normal individuals who gave negative skin tests to ragweed and alder 15 minutes before the experiment. Injection of the filtrate was painless and was usually followed by a triple response (Lewis) which subsided within 30 minutes—one patient developed an erythema at the site of injection which persisted for 3 days leaving a faint pigmentation.

Twenty-four hours later the sites were reinjected with 0.02 ml of ragweed extract (0.01 mg N/ml), alder extract (0.01 mg N/ml) and diluent respectively; at the same time, another test for ragweed sensitivity was made on unprepared skin.

Results. Nasal secretion of 7 (out of 10) ragweed sensitive patients contained ragweed reagins. Alder extract and diluent were negative in all cases; the reagins demonstrated were specific for ragweed. Nasal secretion obtained from normal persons gave negative results. The behavior of reagins other than to ragweed in this particular medium is under investigation. It is felt that these observations might be of significance for a better understanding of the clinical course of pollinosis.

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Society for Experimental Biology and Medicine
ANNUAL REPORT OF THE SECRETARY
June, 1947

The business of the year was transacted by mail and at the annual meetings of the officers and national committees held in Chicago May 19-20, 1947. The following is a concise statement of the principal items:

I. *The Proceedings.*

From October 1946 to April 1947 inclusive, 18 per cent of the manuscripts accepted for publication were preliminary. These articles ranged from ½ to 3½ pages, mode 1½ pages in length. Eighty-two per cent of the articles accepted were "complete." They ranged from 1 to 8 pages in length, mode 3½ pages. The number of articles from each Section was as follows: 80, 26, 24, 24, 23, 19, 15, 12, 11, 8, 6, 5, 2, 1; from areas not organized into Sections, 112.

Delays in Publication. (1) Labor conditions at our printer's have increased the time of publication by about 15 days. Every effort is being made to reduce this delay.

(2) Editors in most instances have been superlatively prompt. There have been exceptions. Plans have been formulated to reduce this source of delay.

(3) The mails have at times been delayed especially at weekends, holidays, and in bad weather (for air mail).

(4) Authors are frequently responsible. The chief items include

a. It is not stated whether manuscript is *preliminary* or *complete.*
b. When complete, *summary* is not supplied.
c. *Title* too long.
d. *Citations* incomplete or incorrect or not given.
e. *Illustrations* will not reproduce adequately or no caption given.
f. *Tables* a duplication of text, or too numerous, or too complex (costly), or no captions.
g. When a manuscript is rejected or radical revision requested by the first editor, it is referred to two other editors. If three agree, author is notified.

Secretaries and authors are requested to note above *a to f* inclusive and help to reduce delays in publication.

II. *Newly Elected Editors.* In accordance with our policy of rotation of editors after five years' service, the following editors were elected to take office September 1947:

Immunology, Dr. F. R. Horsfall, 5 year term
Pathology, Dr. H. P. Smith, 5 year term
Pharmacology, Dr. L. S. Goodman, 3 year term
Physiology, Dr. R. F. Pitts, 5 year term

In selecting editors the Council consider among certain obvious factors (1) who is the best available man in a given subdivision of a science, (2) geographic distribution of editors, (3) least delay in ordinary or air mail services, (4) promptness of new editor, etc., etc.

The Council wish to express their deep appreciation of the splendid and untiring service given by the retiring editors: Doctors Bronfenbrenner, Goldblatt, Long, Lukens, Tillett, Visscher and Welch.

The Council voted to send the PROCEEDINGS without cost for another year to members and subscribers, and to an additional list of institutes, all in the Allied bombed areas, upon their request.

III. *Finances.* Printing costs have increased sharply. This was the chief factor in the deficit of $4206 for the fiscal year ending March 31, 1947. To meet this deficit we used the interest of the Endowment and the Special Surplus Funds. The balance was taken from capital of the Surplus Fund.

To meet the expected larger deficit for the coming fiscal year the Council voted the following to take effect September 1947:

(1) To *increase membership dues* to $5 a year.

(2) To *increase subscription price* to $8.50 a year.

(3) To use the interest of the Surplus Fund.

(4) To *charge* only *5% of cost of text and tables,* of *all* authors.

(5) To *charge 25% of cost for illustrations,* as in the past year.

(6) To *charge 10% above cost for reprints,* as in the past year.

As soon as circumstances warrant these costs to authors will be reduced.

Dr. Cowgill was appointed chairman of a committee to inquire as to the desirability of accepting the highest type of advertisements, and of sustaining members.

IV. *Membership.*

(a) Emeritus members elected were as follows: L. J. Cole, J. Erlanger, J. F. Gaskell, W. L. Holman, F. Knowlton, O. Riddle, E. C. Robertson, C. Voegtlin.

(b) Resignations accepted with regret: L. G. Barth, L. S. Dunn, R. Hober, R. Jackson, F. C. Lee, F. Saunders.

(c) Dropped for arrears: 7 members.

(d) Applications for membership approved: 168.

(e) The time between receipt of, and final action by the National Membership Committee, was reduced to 2½ months.

(f) Section Secretaries are requested to send applications monthly, to make sure that all required information is given in proper form. By these procedures delays should be minimized.

(g) A new form of application was approved. *Sponsors* hereafter *assume certain responsibilities* when they put their signature to the following statement: "In our opinion . . . the . . . nominee meets the minimal requirements of eligibility to membership, according to Art. 9, Sec. 1, for he has published original investigations in experimental biology or experimental medicine . . . , and is actively engaged in experimental research. . . ."

Election of Officers. Since the annual meetings of the Society occurred so late (May 19-20, 1947) the Council authorized a ballot by mail. This ballot was mailed promptly to the members. President Hastings appointed as tellers Doctors Cattell, Harrow, and Severinghaus. They report the following elected:

President—E. M. K. Geiling

Vice-President—E. A. Doisy

Secretary-Treasurer—A. J. Goldforb

Councillors at Large—C. N. H. Long, A. C. Ivy.

Peiping Section. News has come that our members are returning to their laboratories in China. They hope to have their Section functioning again very soon.

South America. The Council approved a plan unanimously supported by our members in South America. Countries where there are three members of the Society may elect one representative to an Inter-Latin American Committee. Countries with 6 or more members may elect two such representatives. The functions of this Committee are (1) to initiate or to receive applications for membership in our Society, and to send such applications with their recommendations to the National Membership Committee. (2) To receive manuscripts from workers in South America for publication in our PROCEEDINGS; to help authors in the preparation thereof; and to transmit their recommendations to the Editorial Board.

There are other functions of this Committee but they do not directly affect the Society. All the functions, it is believed, will tend to raise scientific standards, and to help the Committee to assume certain responsibilities, particularly concerning membership and manuscripts, in those countries represented.

The Council wish again to express their deep appreciation and thanks to

Dr. Emil Baumann of the Montefiore Hospital for continued and splendid indexing of the PROCEEDINGS.

Mr. H. G. Friedman of the General American Investors Co., for continued and most expert advice on our investments.

Mr. Leon Leighton for his readiness to advise on all legal matters pertaining to our investments.

Deaths. The Council records with sorrow the death of the following members: Doctors H. J. Bagg, T. D. Beckwith, C. H. Boissevain, M. Dresbach, M. S. Fine, L. U. Gardner, A. Gelarie, J. S. Horsley, R. W. Lamson, W. P. Larson, H. Plotz, J. P. Schooley, O. T. Schultz, and F. M. Smith.

Past Officers

Date	*President*	*Vice-President*	*Secretary*	*Treasurer*
1903-04	S. J. Meltzer	W. H. Park	W. J. Gies	G. N. Calkins
1904-05	S. J. Meltzer	J. Ewing	" "	" "
1905-06	E. B. Wilson	E. K. Dunham	" "	" "
1906-07	S. Flexner	E. K. Dunham	" "	" "
1907-08	S. Flexner	T. H. Morgan	" "	" "
1908-09	F. S. Lee	T. H. Morgan	" "	G. Lusk
1909-10	F. S. Lee	W. J. Gies	E. L. Opie	" "
1910-11	T. H. Morgan	W. J. Gies	" "	" "
1911-12	T. H. Morgan	P. A. Levene	G. B. Wallace	" "
1912-13	J. Ewing	P. A. Levene	" "	C. Norris
1913-14	J. Ewing	C. W. Field	H. C. Jackson	" "
1914-15	G. Lusk	W. J. Gies	" "	J. R. Murlin
1915-16	G. Lusk	G. N. Calkins	H. C. Jackson	
1916-17	J. Loeb	W. J. Gies	"	"
1917-19	W. J. Gies	J. Auer	"	"
1919-21	G. N. Calkins	G. B. Wallace	"	"
1921-23	G. B. Wallace	J. W. Jobling	"	"
1923-24	H. C. Jackson	J. W. Jobling	V. C. Myers	
1924-25	H. C. Jackson	J. W. Jobling	A. J. Goldforb	
1925-27	J. W. Jobbling	S. R. Benedict	"	"
1927-29	S. R. Benedict	P. Rous	"	"
1929-30	P. Rous	D. Marine	"	"
1930-31	P. Rous	D. J. Edwards	"	"
1931-32	D. J. Edwards	A. R. Dochez	"	"
1932-34	A. R. Dochez	E. L. Opie	"	"
1934-36	E. L. Opie	P. E. Smith	"	"
1936-37	P. E. Smith	E. F. DuBois	"	"
1937-39	H. S. Gasser	J. T. Wearn	"	"
1939-40	J. T. Wearn	C. D. Leake	"	"
1940-41	J. T. Wearn	C. D. Leake	"	"
1941-42	W. deB. MacNider	C. H. Danforth	"	"
1942-43	W. deB. MacNider	C. H. Danforth	"	"
1943-44	A. J. Carlson	A. B. Hastings	"	"
1944-45	A. J. Carlson	A. B. Hastings	"	"
1945-46	A. B. Hastings	E. M. K. Geiling	"	"
1946-47	A. B. Hastings	E. M. K. Geiling	"	"

Sectional Meetings and Membership

Cleveland, Ohio

Chairman: R. A. Shipley Secretary: M. Miller Members: 56

Meetings: Western Reserve University, October 11, 1946
November 8, 1946
December 13, 1946
January 10, 1947
February 14, 1947
March 14, 1947
May 9, 1947

District of Columbia

Chairman: M. X. Sullivan Secretary: F. S. Daft Members: 97

Meetings: George Washington University, December 5, 1946
February 6, 1947
March 6, 1947
April 3, 1947

Illinois

Chairman: F. C. McLean Secretary: H. Necheles Members: 205

Meetings: University of Illinois Med., November 19, 1946
Northwestern University, March 4, 1947
Michael Reese Hospital, January 21, 1947

Iowa

Chairman: E. D. Warner Secretary: E. L. DeGowin Members: 41
Meetings: State University of Iowa, December 17, 1946

Minnesota

Chairman: R. G. Green Secretary: B. Campbell Members: 70
Meetings: University of Minnesota, October 9, 1946
November 15, 1946
December 11, 1946
January 8, 1947
February 19, 1947
March 12, 1947
April 11, 1947
May 28, 1947

Missouri

Chairman: L. R. Jones Secretary: C. F. Cori Members 64

New York

Chairman: W. S. Root Secretary: W. M. Copenhaver Members: 519
Meetings: New York Academy of Medicine, December 11, 1946
February 5, 1947
April 9, 1947

Pacific Coast

Chairman: M. Kleiber Secretary: J. J. Eiler Members: 125
Meetings: University of California, October 2, 1946
February 12, 1947
Stanford University, April 26, 1947

Peiping, China

Rocky Mountain

Chairman: A. R. Buchanan Secretary: J. A. Anderson Members: 40
Meetings: Colorado A. and M. College, October 19, 1946
University of Colorado, February 22, 1947
University of Utah, March 15, 1947

Southern

Chairman: R. G. Smith Secretary: C. Reynolds Members: 58
Meetings: Medical College of Alabama, December 7, 1946
Tulane University, February 6, 1947
Louisiana State University, May 9, 1947

Southern California

Chairman: J. W. Mehl Secretary: A. J. Haagen-Smit Members: 60
Meetings: California Institute of Technology, November 18, 1946
University of Southern California, March 14, 1947

Southwestern

Chairman: R. H. Rigdon Secretary: A. A. Hellbaum Members: 69
Meetings: University of Texas, October 26, 1946
Southwestern Medical College, March 29, 1947

Western New York

Chairman: J. B. Sumner Secretary: L. L. Varnes Members: 73
Meetings: University of Rochester, October 26, 1946
February 22, 1947

Wisconsin

Chairman: H. P. Rusch Secretary: W. H. McShan Members: 53
Meetings: University of Wisconsin, November 21, 1946
March 25, 1947

MEMBERSHIP

Members, March 31, 1946	2006	
Elected during year	227	
		2233
Resignations	6	
Arrears	7	
Deaths	20	
		33
Total Membership, March 31, 1947		2200

Membership:	1937	1947
	1354	2200

Subscriptions, March 31, 1947 1436

ANNUAL REPORT OF THE TREASURER
April 1, 1946-April 1, 1947

President Hastings appointed Doctors Greenwald, Gregersen and Hinsey as Auditing Committee to examine the accounts of the Treasurer. This Committee requested Mr. Alexander Dolowitz, C.P.A., to go over the books of the Society and report to the Committee. The following is Mr. Dolowitz's abbreviated statement:

Society for Experimental Biology and Medicine
STATEMENT OF ASSETS AND LIABILITIES
MARCH 31, 1947

Assets

Cash in bank	$ 4,946.42	
Investments—Surplus Fund (Special)	32,475.19	
" —Endowment Fund	22,554.65	
Accounts receivable	2,503.46	
Total assets		$62,479.72

Liabilities

Withholding taxes	$ 123.50	
Miscellaneous	212.72	
Total liabilities		$ 336.22
Net worth		$62,143.50

Endowment Fund

N. Y. Title and Mortgage Co.	$ 3,960.00	
Title Guarantee and Trust Co.	114.01	
Lawyers Mortgage Co.	83.10	
Bowery Savings Bank	2,856.48	
R. R. Federal Savings and Loan	79.56	
U. S. Government Bonds	12,200.00	
Industrial Bonds	1,848.62	
Manufacturers Trust Co.—Cash	1,412.88	
Total		$22,554.65

Surplus Fund (Special)

Harlem Savings Bank	$ 3,452.79	
U. S. Government Bonds	26,295.71	
Industrial Bonds	2,726.69	
Total		$32,475.19

Society for Experimental Biology and Medicine

Statement of Income and Disbursements

April 1, 1946 to March 31, 1947

INCOME

Dues		$ 8,093.48	
Subscriptions	$11,124.30		
Reprints	3,628.85		
Excess Space	7.37		
Cuts	99.50		
Back Numbers	1,946.66		
		16,806.68	
Interest—Special Accounts		2.08	
Miscellaneous		200.15	
Total Income			$25,102.39

DISBURSEMENTS

Office supplies, telephone and postage		$ 2,206.64	
Cost of printing	$16,924.36		
Cost of reprints	3,131.35		
Cuts	1,801.85		
Storage and insurance	116.25		
Refunds	271.50		
Back numbers (purchases)	222.55		
		22,467.86	
Salaries		4,004.24	
Miscellaneous		682.35	
Total disbursements			29,361.09
Excess of disbursements over income			$ 4,258.70